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ONE CARBON METABOLISM IN ISOLATED PEA MITOCHONDRIA

by

© MICHAEL THOMAS CLANDININ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled 'One Carbon Metabolism in Isolated Pea Mitochondria' submitted by Michael Thomas Clandinin in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Biochemistry.

Edward A. Cass
Supervisor

David D. Cass

G. Pharr

Asad Ahmed

External Examiner

Date. October 15, 1973

ABSTRACT

Mitochondria were extracted from 4-day-old pea cotyledons and purified on a sucrose density gradient. Microbiological assay of the purified mitochondrial fraction using *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043) and *Pediococcus cerevisiae* (ATCC 8081) revealed a discrete pool of conjugated and unconjugated derivatives of tetrahydropteroylglutamic acid. Solubilization and chromatographic studies of the mitochondrial fraction demonstrated the presence of formylated and methylated derivatives, 10-formyltetrahydropteroylmonoglutamic acid, 5-formyltetrahydropteroylmonoglutamic acid and 5-formyltetrahydropteroyldiglutamic acid being the major derivatives present. The principal mitochondrial pteroylglutamates were labelled when dry seeds were imbibed in [2-^{14}C]pteroylglutamic acid and [*methyl*-^{14}C]-5-methyltetrahydropteroylmonoglutamic acid. The ability of isolated mitochondria to catalyze oxidation and reduction of tetrahydropteroylglutamic acid derivatives was demonstrated in feeding experiments in which [^{14}C]-HCHO, [3-^{14}C]serine, sodium [^{14}C]formate, [*methyl*-^{14}C]-5-methyltetrahydropteroylmonoglutamic acid or [2-^{14}C]-glycine served as one-carbon donors. In addition, ^{14}C was incorporated into free amino acids related to one-carbon metabolism.

The kinetics of the glycine decarboxylase reaction and its potential as a C-1 source were examined by the glycine-bicarbonate exchange reaction. The enzyme was found to have a K_m for glycine of 1.8 mM, and a K_m for bicarbonate of 12.5 mM. Reduced pyridine

nucleotides were found to inhibit the exchange reaction while stimulation of decarboxylation occurred in the presence of NAD with a K_m for NAD of 47 mM.

The ability of isolated mitochondria to synthesize methionine from ^{14}C labelled pteroylglutamate derivatives in the presence of homocysteine was also investigated. A homocysteine-dependent methyltransferase utilizing 5-methyltetrahydropteroylglutamic acid as the methyl donor was shown to be localized in this organelle. Some catalytic properties of this enzyme were compared with those of a similar enzyme present in tissue homogenates. The isolated mitochondria were also capable of synthesizing S-adenosyl-L-methionine from methionine and ATP. Enzymes catalyzing the conversion of homoserine to homocysteine were also detected in mitochondrial extracts.

It is concluded that H_4PteGlu derivatives play highly significant metabolic roles in the mitochondria of this species.

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LIST OF ABBREVIATIONS*

** 10-HCO-H_4PteGlu	:	N^{10}-formyltetrahydropteroylmonoglutamate
*** PteGlu	:	pteroylmonoglutamate
SAM	:	S-adenosyl-L-methionine
µCi	:	microcurie
cpm	:	counts per minute
ATP	:	adenosine triphosphate
TRIS	:	tris(hydroxymethyl)aminomethane
EC	:	Enzyme Commission
ATCC	:	American Type Culture Collection
DEAE-cellulose	:	diethylaminoethyl-cellulose
EDTA	:	disodium ethylenediaminetetra-acetic acid
PALP	:	pyridoxal-5'-phosphate
ADP	:	adenosine diphosphate
t-RNA	:	transfer RNA
TES	:	N-tris(hydroxymethyl)methyl-2-aminomethane sulphonic acid
TPP	:	thiamine pyrophosphate
NAD(P)	:	nicotinamide adenine dinucleotide (phosphate)
SMM	:	S-methyl-L-methionine
DTT	:	dithiothreitol

* Other abbreviations commonly utilized in the text are given in the format acceptable for publication in the *Biochemical Journal*.

** The abbreviations used for pteroylglutamic acid and its derivatives are those suggested by the IUPAC-IUB Commission as listed in the *Biochemical Journal* 102: 15 (1967).

*** This abbreviation is used in the figures to designate pteroyl-glutamate derivatives in general and is synonymous with the term "folate" used in earlier literature.

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INTRODUCTION

Historical

From early studies it is clear that a wide variety of biological materials contain pteroylglutamate derivatives (Blakley, 1969; Butterworth *et al.*, 1963; Santini *et al.*, 1964; Iwai and Nakagawa, 1958a, 1958b). Following this discovery, attention was centred on techniques for isolation and characterization of these compounds (Bakerman, 1961; Silverman *et al.*, 1961; Sotobayashi *et al.*, 1964; Iwai *et al.*, 1959). Techniques of prime importance in this area now commonly involve column chromatography and differential microbiological assay (see review by Blakley, 1969). In assay of plant tissues, however, no information was obtained concerning the concentrations of 5-CH_3-H_4PteGlu but, it was demonstrated that reduced derivatives often existed as glutamyl peptides. Later more detailed investigations of higher plants (Roos *et al.*, 1969; Shah and Cossins, 1970; Roos and Cossins, 1971; Rohringer *et al.*, 1969; Cossins and Shah, 1971) involving modifications of these techniques, together with differential microbiological assays based on *Lactobacillus casei* growth response have revealed that 5-methyl and conjugated derivatives are commonly the principal components of the pteroylglutamate pool.

Derivatives of PteGlu act as coenzymes in many metabolic reactions. Such PteGlu coenzymes are primarily concerned with the transfer of

one-carbon units at the oxidation levels of formate, formaldehyde and methanol, and in transforming these from one oxidation level to another. The first metabolically active PteGlu derivative to be characterized was 5-HCO-H_4PteGlu (Sauberlich and Baumann, 1948; Broquist *et al.*, 1949). Later the significance and identity of 10-HCO-H_4PteGlu, 5,10-CH_2=H_4PteGlu, 5-HCNH-H_4PteGlu, 5,10-CH_2-H_4PteGlu and 5-CH_3-H_4PteGlu was also determined (Bakerman, 1961; Blakley, 1960; Sakami and Ukstins, 1961; Keresztesy and Donaldson, 1961; Gupta and Huennekens, 1967; Sharadchandra *et al.*, 1955; Jaenicke, 1956; Kisliuk, 1957; Blakley, 1957, 1959, 1960; Rabinowitz and Pricer, 1956; Raven and Jaenicke, 1953; Tabor and Rabinowitz, 1956; Tabor and Mehler, 1954).

Sources of C-1 Units

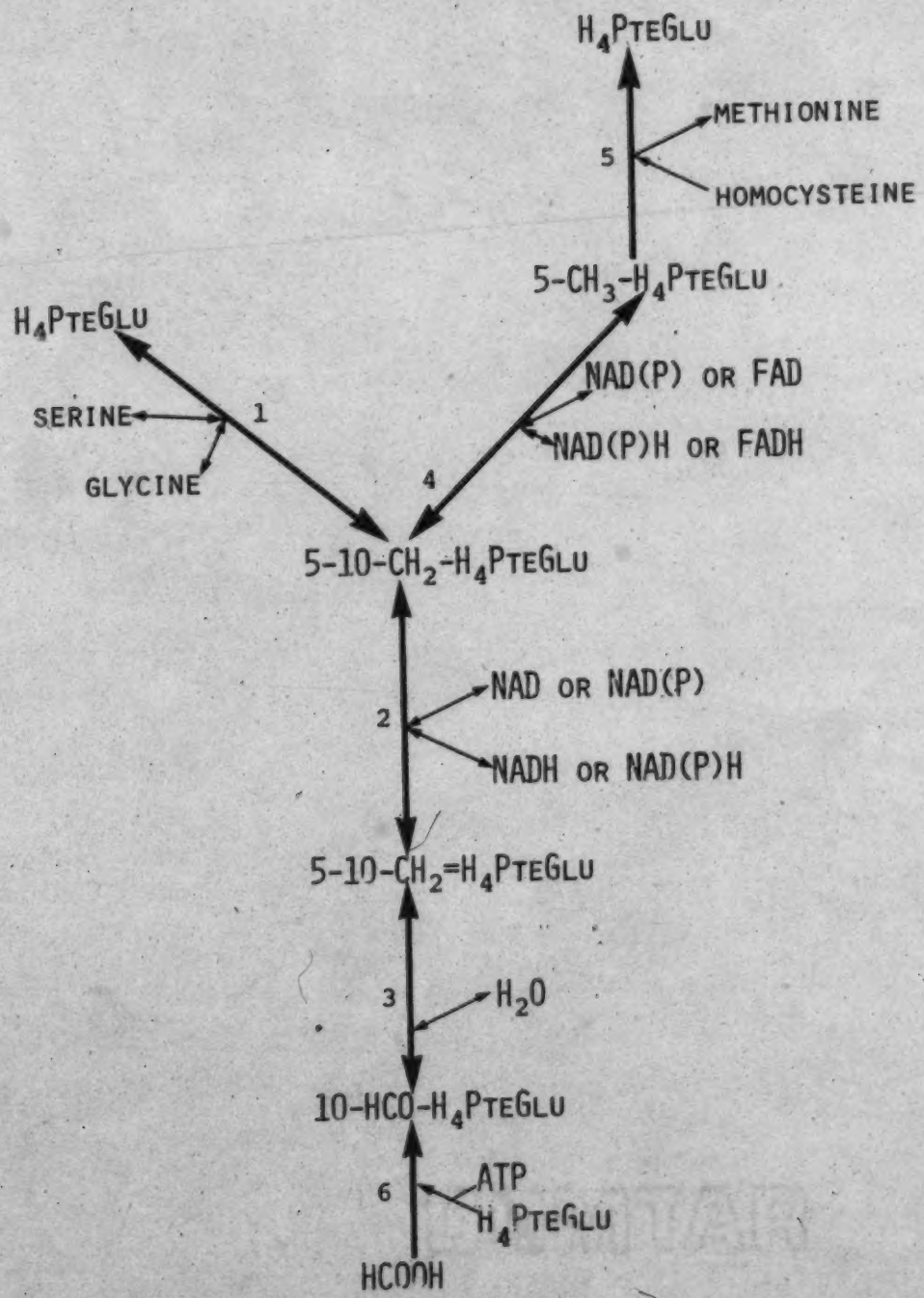
The one-carbon unit of these metabolically active derivatives can be oxidized or reduced through enzyme mediated reactions as illustrated in Scheme 1. The major biological source of this one-carbon unit is believed to be serine with formate and glycine as other potential donors.

Serine hydroxymethyltransferase (Scheme 1, reaction 1), which catalyzes the reversible formation of 5,10-CH_2-H_4PteGlu and glycine from serine, is considered to be the major C-1 donor to the pteroylglutamate pool. The enzyme has been reported from several tissues (Alexander and Greenburg, 1956; Huennekens *et al.*, 1957; Schirch and Mason, 1962; Wang and Burris, 1965; Cossins and Sinha, 1966; De Boiso and Stoppani, 1967; Martinez-Carrion *et al.*, 1972; Schirch and Gross, 1968; Mazelis and Lui, 1967; Uyeda and Rabinowitz, 1968; Schirch and Jenkins, 1964; Schirch and Diller, 1971; Clandinin and Cossins, 1972).

SCHEME 1

The major reactions for production of C-1 units in plant tissues

Enzymes	EC Number	Reaction
L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase	2.1.2.1	1
5-methyltetrahydrofolate NADP oxidoreductase	1.5.1.5	2
5,10-methenyltetrahydrofolate 5-hydrolase	3.5.4.9	3
5,10-methylenetetrahydrofolate NADP oxidoreductase	1.1.1.68	4
5-methyltetrahydrofolate: homocysteine methyltransferase	2.1.99	5
N^{10}-formyltetrahydrofolate synthetase	6.3.4.3	6


H4PteGlu
METHIONINE
5
HOMOCYSTEINE
5-CH3-H4PteGlu
H4PteGlu
NAD(P) OR FAD
SERINE
1
NAD(P)H OR FADH
GLYCINE
4
5-10-CH2-H4PteGlu
NAD OR NAD(P)
2
NADH OR NAD(P)H
5-10-CH2=H4PteGlu
3
H2O
10-HCO-H4PteGlu
6
ATP
H4PteGlu
HCOOH

In several tissues, the ATP dependent activation of formate, in the presence of H_4PteGlu, to 10-HCO-H_4PteGlu has also been demonstrated (Blakley, 1969). The normal physiological role of formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase (ADP) EC 6.2.4.3) which mediates the following reaction, is thought to be the synthesis of metabolically active one-carbon units from formate (Goldthwait and Greenberg, 1955; Greenberg *et al.*, 1955; Whiteley *et al.*, 1958).

1. $$HCOOH + (-)H_4PteGlu + MgATP \xrightarrow{NH_4^+} (-)10\text{-}HCO\text{-}H_4PteGlu + MgADP + P_i$$

In microorganisms such as *Clostridium*, the activity of this enzyme is consistent with a limited role as it constitutes only a small proportion of the protein in soluble extracts (Rabinowitz and Pricer, 1958, 1962; Nowak and Himes, 1971; Curthoys and Rabinowitz, 1971). In *Escherichia coli*, *Peptococcus glycinophilus* and mammalian liver, glycine is split by a reaction (Scheme 2, p. 11) involving H_4PteGlu and PALP yielding as products CO_2, NH_3 and 5,10-CH_2-H_4PteGlu (Greenberg *et al.*, 1955; Kawosaki *et al.*, 1966; Sato *et al.*, 1969a,b; Motokawa and Kikuchi, 1969a,b; Motokawa and Kikuchi, 1971; Yoshida and Kikuchi, 1970; Yoshida and Kikuchi, 1971; Osborne *et al.*, 1960; Klein and Sagers, 1966a,b, 1967a,b). Tracer studies involving [2-^{14}C]glycine feeding have indicated that a similar reaction may occur in plants (Sinha and Cossins, 1964; Cossins and Sinha, 1966; McConnell, 1964; Clandinin and Cossins, 1972) as proposed in the glycollate pathway (Wang and Burris, 1965; Miflin *et al.*, 1966; Bruin *et al.*, 1970). The significance of this reaction will be discussed later.

Other sources of one-carbon units may include formaldehyde

(Seigel and Lafaye, 1950; Mitoma and Greenberg, 1952), the methyl groups of choline (Kuchoffer, 1951; Sakami, 1949; Siekevitz and Greenberg, 1950), acetone (Sakami, 1950), dimethylglycine and sarcosine (Mackenzie, 1950; Mackenzie and Abels, 1956; Mackenzie and Frisell, 1958), and the formimino groups of formiminoglutamic acid (Tabor and Wyngarden, 1959) and formiminoglycine (Rabinowitz and Pricer, 1956).

Interconversion of H_4PteGlu Derivatives

The enzymes referred to in this section are those utilizing derivatives of H_4PteGlu as substrates and are summarized in Scheme 1 (p. 3). 5,10-CH_2-H_4PteGlu, formed from free formaldehyde, C-3 of serine (Scheme 1, reaction 1) or C-2 of glycine (Scheme 2, reaction 2, p. 11) may be subject to oxidation or reduction. In the presence of NADP and 5,10-methylene-H_4PteGlu dehydrogenase oxidation may occur to 5,10-CH=H_4PteGlu (Scheme 1, reaction 2) (Osborne and Huennekens, 1957; Uyeda and Rabinowitz, 1967; Wong and Cossins, 1966; Cossins *et al.*, 1970). Further hydration usually occurs to 10-HCO-H_4PteGlu (Scheme 1, reaction 3) with the accompanying addition of H_2O by cyclohydrolase (Rabinowitz and Pricer, 1956; Tabor and Rabinowitz, 1956; Tabor and Syngarden, 1959). The partial purification of 5,10-CH_2-H_4PteGlu dehydrogenase from several sources has been accomplished and some of its properties investigated (Ramasastri and Blakley, 1964; Donaldson *et al.*, 1965; Yeh and Greenberg, 1965; Dalal and Gots, 1967; Cossins *et al.*, 1970).

The reduction of 5,10-CH_2-H_4PteGlu to 5-CH_3-H_4PteGlu (Scheme 1, reaction 4) is dependent upon the presence of reduced pyridine nucleotides and 5,10-CH_2-H_4PteGlu reductase. The reverse reaction can be

stimulated by the addition of electron acceptors such as menadione (Donaldson and Keresztesy, 1962). Recently it has been shown that reduction of 5,10-CH_2-H_4PteGlu is facilitated by adding FAD in the presence of a diaphorase and reduced pyridine nucleotide to the reducing system (Katzen and Buchanan, 1965; Clandinin and Cossins, 1972). Presumably $FADH_2$ synthesized in such a system is preferentially utilized for the reduction of 5,10-CH_2-H_4PteGlu. Where 5,10-CH_2-H_4PteGlu reductase has been purified up to 100 fold it was clearly demonstrated that $FADH_2$ is specifically required as the reductant and NADH can only serve as the source of hydrogen when present with FAD and a lipoamide dehydrogenase (Katzen and Buchanan, 1965).

The enzymic synthesis of 5-HCO-H_4PteGlu from 5,10-CH=H_4PteGlu has been reported by Peters and Greenberg (1957) and Greenberg *et al.* (1965). The enzyme catalyzing this reaction has been called 5-HCO-H_4PteGlu cyclodehydrase.

Cyclodeaminase activity catalyzing the deamination of 5-HCNH-H_4PteGlu to form 5,10-CH=H_4PteGlu has been purified from *C. cylindrosporum* and mammalian liver (Rabinowitz and Pricer, 1956; Uyeda and Rabinowitz, 1967; Tabor and Rabinowitz, 1956; Tabor and Wyngarden, 1959). It is not clear yet whether this activity is catalyzed by a separate enzyme, as Uyeda and Rabinowitz (1967) have suggested that such deaminase activity could not be separated experimentally from the cyclohydrolase active protein present.

Involvement of H_4PteGlu Derivatives in Purine, Pyrimidine, Amino Acid and Protein Biosynthesis

Rabinowitz and Pricer (1957) have demonstrated that H_4PteGlu

derivatives are involved in the biosynthesis of carbons 2 and 8 of the purine ring. H_4PteGlu derivatives are also involved in the introduction of one-carbon units in the synthesis of thymine, 5-hydroxymethylcytosine and 5-methyluridine (Whittaker and Blakley, 1961; Flaks and Cohen, 1959; Maley, 1962).

Several very critical biological roles are fulfilled by derivatives of H_4PteGlu. Recently it has been discovered that formyl methionine plays a primary role in initiating the synthesis of polypeptide chains. The formyl donor has been shown to be 10-HCO-H_4PteGlu. The enzyme responsible for the formylation of methionyl-$tRNA^{fmet}$ has been purified (Marikoshi and Doi, 1967; Dickerman *et al.*, 1967).

5-CH_3-H_4PteGlu$_{(n)}$ is utilized in all organisms investigated as the CH_3 donor in the transmethylation reactions which result in the biosynthesis of methionine from homocysteine and 5-CH_3-H_4PteGlu (Weissbach *et al.*, 1963; Kerwar *et al.*, 1966; Dodd and Cossins, 1970; Burton and Sakami, 1969). Dodd and Cossins (1969, 1970) have concluded that these derivatives are important as methyl donors in the *de novo* synthesis of methionine during seed germination. This latter amino acid is utilized in plant tissue as a methyl donor after it has been activated by ATP in the following reaction,

2. Methionine + ATP $\xrightarrow{Mg^{++}}$ SAM + pyrophosphate + P_i

(Mudd, 1960; Davies, 1966). SAM is then utilized as a source of methyl groups for synthesis of a variety of compounds including lignin, pectin, chlorophyll and quinones (Byerrum *et al.*, 1954; Sato *et al.*, 1958; Radmer and Bogorad, 1967; Threlfall, 1967).

Localization and Interconversion of H_4PteGlu Derivatives

Iwai *et al.* (1967) and Okinaka and Iwai (1970) have demonstrated that several key enzymes of pteroylglutamate synthesis are localized in the mitochondria of plants. It has been further suggested that most pteroylglutamate precursors including dihydropteroylglutamic acid are synthesized in the mitochondrion and are transported to the cytoplasm where further reduction and addition of one-carbon units is thought to occur (Okinaka and Iwai, 1970). In contrast to this suggestion, Wang *et al.* (1967) have shown that 10-HCO-H_4PteGlu is the major constituent of the pteroylglutamate pool of rat liver mitochondria. These authors have suggested, on the basis of enzyme studies, that the *de novo* synthesis of methyl groups and interconversion of 10-HCO-H_4PteGlu and 5,10-CH_2-H_4PteGlu do not occur in rat liver mitochondria. Sankar *et al.*(1969) have also reported an association of uncharacterized pteroylglutamates with mitochondria isolated from mouse liver. In preliminary studies with isolated plant mitochondria, derivatives of H_4PteGlu were detected but their synthesis and physiological significance was not examined (Shah *et al.*, 1970). More detailed examination of this localization revealed that a considerable metabolism of H_4PteGlu derivatives occurs in isolated mitochondria (Clandinin and Cossins, 1972).

Recent work by Tolbert and co-workers (Tolbert, 1962; Tolbert *et al.*, 1968; Tolbert *et al.*, 1969; Kisaki and Tolbert, 1969; Bruin *et al.*, 1970) has resulted in the proposal of schemes for the utilization of glyoxylate, glycollate and serine which involve chloroplastic, mitochondrial and peroxisomal compartments within the cell. As these reactions are thought to involve at least one reaction of one-carbon

metabolism, it is clear that pteroylglutamates and enzymes catalyzing their interconversion could be associated to some extent with these cellular fractions. Earlier studies from this laboratory (Shah and Cossins, 1969; Shah *et al.*, 1970; Shah and Cossins, 1970; Cossins and Shah, 1971) have substantiated the involvement and localization of pteroylglutamates in the one-carbon metabolism of pea chloroplasts.

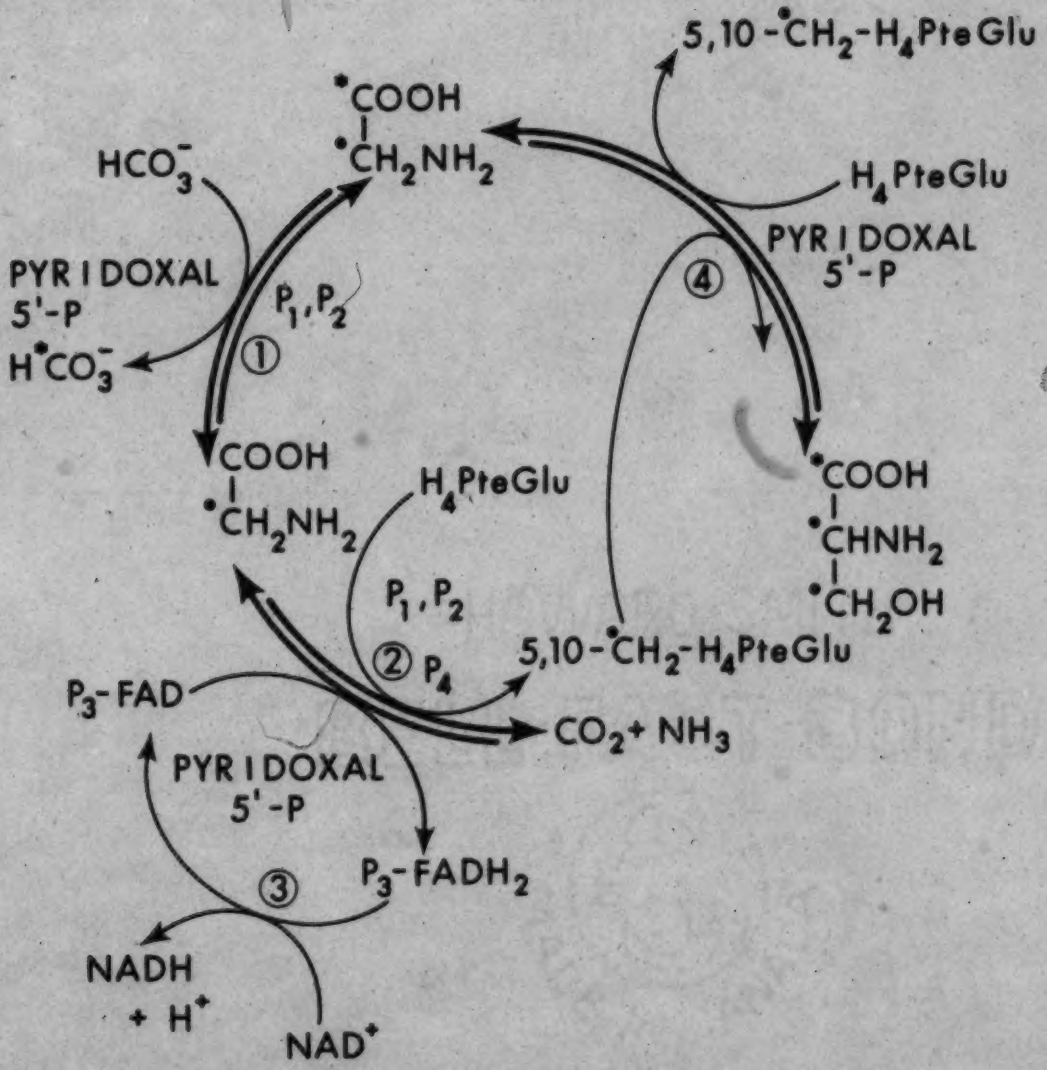
Glycine and its Relationship to H_4PteGlu Metabolism

As previously mentioned, it has been clearly demonstrated by several lines of evidence that the α-carbon of glycine is converted to the β-carbon of serine in rat liver (Kikuchi and co-workers: Kawasaki *et al.*, 1966; Sato *et al.*, 1969a,b; Motokawa and Kikuchi, 1969a,b; Motokawa and Kikuchi, 1971; Yoshida and Kikuchi, 1970; Yoshida and Kikuchi, 1971). Similar data have been obtained for bacteria (Osborne *et al.*, 1960; Nash, 1953; Van Slyke *et al.*, 1971) and plants (Bruin *et al.*, 1970; Kisaki *et al.*, 1971; Clandinin and Cossins, 1972; Prather and Sisler, 1972). Extensive study and purification of the enzyme system from *Peptococcus glycinophilus*, which catalyzes the labilization of the glycine carboxyl group, has been accomplished by Klein and Sagers (1966a,b, 1967a,b). These workers have also made detailed observations on the properties of the four protein subunits (P_1, P_2, P_3, P_4) involved in the overall reaction (Scheme 2, p. 11).

Kikuchi and co-workers have shown that the most significant pathway of glycine metabolism in human (Yoshida *et al.*, 1969; Tado *et al.*, 1969) and rat livers (Yoshida and Kikuchi, 1970) is the direct cleavage of glycine to form 5,10-CH_2-H_4PteGlu, CO_2 and ammonia followed by subsequent oxidation of the C-1 unit to CO_2 in the mitochondria.

These authors consider the physiological catabolism of serine in rat liver, to proceed mainly by way of preliminary elimination of the C-3 of serine at the hydroxymethyl level of oxidation to produce 5,10-CH_2-H_4PteGlu and glycine followed by further oxidation to yield CO_2 as the final product. In contrast, Reichert *et al.* (1962) have demonstrated considerably higher activity of the glycine cleavage reaction in avian liver than in rat liver, but very little oxidation of the α-carbon of glycine to CO_2 could be shown. Yoshida and Kikuchi (1971) have demonstrated that in avian liver the oxidation of 5,10-CH_2-H_4PteGlu to CO_2 is severely limited by low activity of 10-formyl-H_4PteGlu NADP+: oxidoreductase and have further suggested that the C-1 unit is drawn off for other syntheses in this tissue such as positions 2, 5 and 8 of uric acid.

In plant tissues, Kisaki *et al.* (1971) have reported the localization of glycine decarboxylase in the mitochondria of photosynthetic tissue, a finding quite compatible with the pathways of glycollate metabolism proposed by Tolbert and co-workers (Tolbert, 1962; Tolbert *et al.*, 1968; 1969; Kisaki and Tolbert, 1969; Bruin *et al.*, 1970). These latter workers have proposed that the physiological significance of glycine decarboxylase in the mitochondrion of photosynthetic tissues is in the generation of CO_2 of photorespiration and subsequent transhydroxymethylation to produce serine. In direct contrast to this proposal, Zelitch (1972) does not believe that the stoichiometry of the pathway could release sufficient CO_2 to account for the known rates of photorespiration and therefore suggests that glycollate is a more immediate precursor of the CO_2 arising in photorespiration. Consistent with this, Zelitch (1972) has demonstrated



SCHEME 2

Reactions of Glycine Cleavage

Interrelationships and reactions of the four protein subunits (P_1, P_2, P_3, P_4) in the glycine cleavage sequence, described by Klein and Sagers (1966a,b; 1967a,b), in *Peptococcus glycinophilus*.

that glycine, in tobacco leaf tissue, is not a mandatory precursor of the large quantities of CO_2 arising during photorespiration.

In germinating cotyledons (a non-photosynthetic tissue) Clandinin and Cossins (1972) have demonstrated that [2-^{14}C]glycine is apparently split when fed to isolated mitochondria with subsequent incorporation of radioactivity into serine and methionine as well as labelling of formyl and methyl derivatives of tetrahydropteroylglutamic acid. Similarly these authors have demonstrated the incorporation of radioactivity from [3-^{14}C]serine into glycine by isolated mitochondria, indicating that the glycine splitting reaction in this tissue may operate to some extent in the direction of glycine synthesis.

The Present Investigation

Numerous aspects of the C-1 metabolism of germinating *Pisum sativum* L. cultivar Homesteader seeds have been investigated in this laboratory (*e.g.* Cossins and Sinha, 1965; Wong and Cossins, 1966; Dodd and Cossins, 1968, 1969, 1970; Roos *et al.*, 1969; Cossins *et al.*, 1970; Roos and Cossins, 1971; Shah and Cossins, 1970; Sengupta and Cossins, 1971). Consequently this tissue was selected for the present investigation.

In order to assess the possible involvement of mitochondria in the one-carbon metabolism of plant tissues, the occurrence and interconversion of pteroylglutamate derivatives in this organelle, isolated from germinating pea cotyledons, have been examined. Several reactions that are likely involved in the generation of methyl groups for the biosynthesis of methionine were also examined in the present studies. As the occurrence and physiological significance of glycine

cleavage in isolated mitochondria is not clear, the present work has examined some of the properties of this glycine decarboxylase and its bicarbonate exchange reaction, in relation to the pteroylglutamate pool. The reaction as a potential source of C-1 units has also been considered.

Some aspects of the regulation of the compartmented biosynthesis of 5-CH_3-H_4PteGlu will be discussed. The subsequent transmethylation reactions involved in the biosynthesis of methionine and S-adenosyl-L-methionine within the mitochondria are also examined.

MATERIALS AND METHODS

MATERIALS

Chemicals. [^{14}C]Formaldehyde, sodium [^{14}C]formate, [2-^{14}C]-PteGlu acid, [*methyl*-^{14}C]-5-CH_3-H_4PteGlu, [2-^{14}C]glycine and L-[3-^{14}C]-serine were purchased from Amersham-Searle Corporation, Des Plaines, Illinois, U.S.A.. The purity of these labelled compounds was checked by column and thin layer chromatographic techniques described in the Methods section. Other chemicals, of the highest quality commercially available, were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A., Sigma Chemical Company, St. Louis, Mo., U.S.A., and Fisher Scientific Co., Edmonton. Tetrahydrofolic acid was purchased exclusively from Sigma Chemical Company. Scintillation grade 2,5-diphenyloxazole (PPO) and 1,4-*bis*-[4-methyl-5-phenyloxazol-2-yl)-benzene (dimethyl POPOP) were purchased from Nuclear-Chicago, Des Plaines, Illinois, U.S.A.

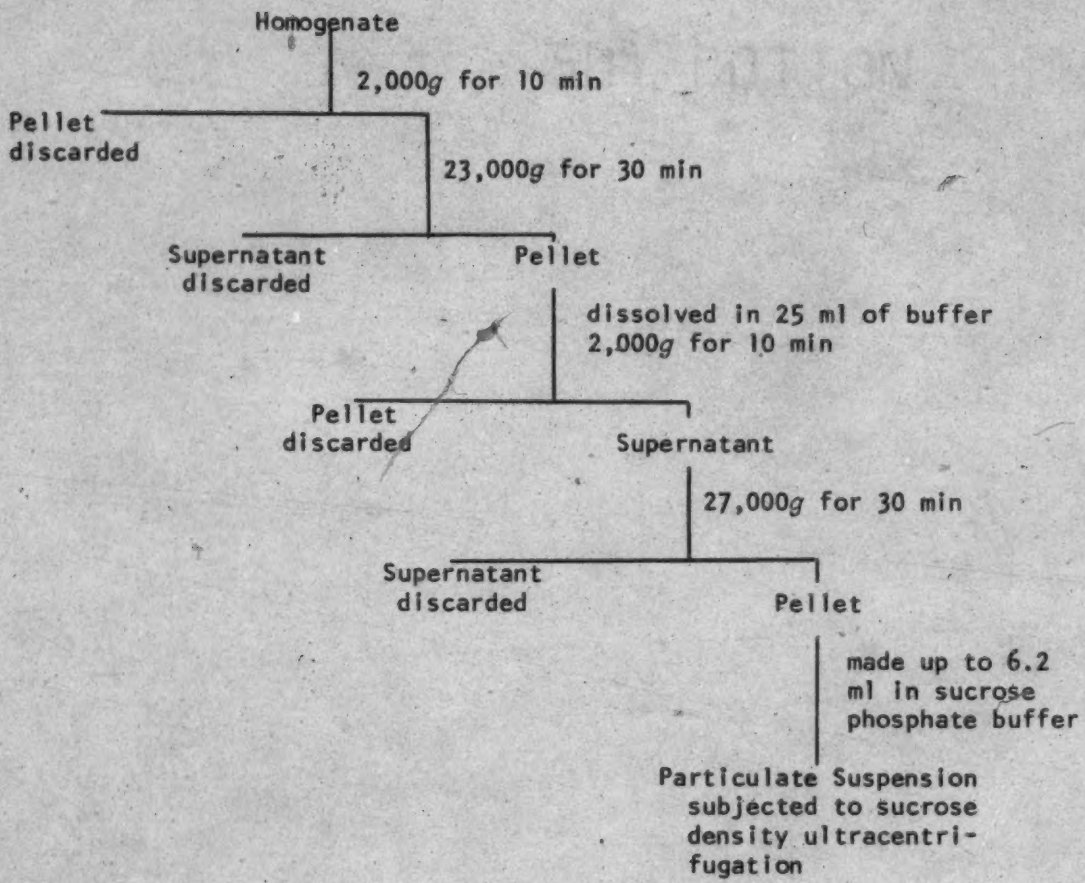
Plant material. Seeds of *Pisum sativum* L. cultivar Homesteader were soaked in deionized water at 25°C. After 30 min, seeds that had begun to imbibe water were discarded and the remainder were allowed to complete imbibition for a further period of 7 h. Fully imbibed seeds were then selected and germinated in moist vermiculite in darkness at 25°C for 88 h. Moist vermiculite was added to the flat to approximately two inches above the level of the seed to ensure

complete coverage of the radicle during germination. After germination, the radicle, root and testa were removed from the seed leaving only the cotyledon, which was then rinsed with distilled water, weighed and chilled in a beaker placed on crushed ice.

METHODS.

Homogenization and preparation of particulate fraction. All operations were carried out at 2-4°C. Samples of cotyledons (150 g) were homogenized by hand in a meat grinder with 300 mls of 0.1 M potassium phosphate buffer (pH 7 at 5°C) containing 0.5 M sucrose. Radioactive samples were homogenized with a mortar and pestle. The homogenate was passed through six layers of cheesecloth and subjected to differential centrifugation as shown in Scheme 3 (p. 16).

The final pellet was suspended in the extraction buffer to a final volume of 6.2 mls and aliquots of 1 ml were layered on each of six discontinuous sucrose density gradients. Each density gradient consisted of nine layers and was prepared several hours earlier at 4°C by pipetting in sequence: 1 ml of 77.2%, 0.5 ml of 67.6%, 1 ml of 64.5%, 3 ml of 61.5%, 3 ml of 58.5%, 0.5 ml of 52.7%, 0.5 ml of 47%, 0.5 ml of 40%, and 2 ml of 26.4% (w/v) sucrose. The gradients, contained in a SW-40 Spinco rotor, were centrifuged at 40,000 rev/min (199,000*g* at $R_{av.}$) for 190 min in a Beckman Spinco Model L2-65B Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California, U.S.A.) and decelerated without braking. Preliminary studies utilized a continuous sucrose density gradient (20% to 60%) centrifuged at 23,000 rpm in a S.W. 25./rotor for 4 h. Fractions were collected from the bottom of the tube in a cold room at 2°C, as shown in Table 1. Subsequent



Scheme 3

Preparation of particulate fractions by differential centrifugation

Table 1. *Protein content, volume and sucrose concentration of fractions collected from the sucrose density gradient*

Fraction	Volume of fraction (mls)	Sucrose Conc. (mol)	Protein % Distribution
1	1.0	2.26	7.1
2	1.5	1.91	9.3
3	3.0	1.79	13.5
4	3.0	1.71	32.7
5	1.0	1.46	22.8
6	0.5	1.14	2.9
7*	3.0	0.68	11.6

* Uppermost fraction of the gradient.

The percentage distribution of protein within the gradient is the mean of three separate analyses, run in duplicate. Fraction 5 was found to contain a mean of 253.9 µg of protein/g fresh weight of cotyledons extracted.

enzyme assays were performed immediately. Protein was measured by the method of Lowry *et al.* (1951) using crystalline egg albumin as a reference standard.

Enzyme assays. Spectrophotometric assays were conducted using a Beckman DB recording spectrophotometer (Beckman Instruments Inc., Palo Alto, California, U.S.A.).

Fumarase (EC 4.2.1.2) was assayed by the method of Massey (1955), which is based on the high ultraviolet absorption of fumarate, malate by comparison having an almost negligible ultraviolet absorption. The complete assay system contained: enzyme, 0.017 M sodium fumarate and 0.033 M potassium phosphate buffer (pH 7.3), in a total volume of 3 mls. One unit of activity is defined as the amount of enzyme causing an initial rate of change of log (I_0/I) at 300 nm of 0.01 per min at 20°C and pH 7.3.

Succinic dehydrogenase (EC 1.3.99.1) was assayed by the colorimetric method of Hiatt (1961). This method follows the rate of reduction of sodium 2,6-dichlorophenolindophenol utilizing N-methyl phenazonium sulphate as the final electron acceptor. The complete assay system (final volume 3 mls) contained: 0.05 M potassium phosphate buffer (pH 7.4), 0.04 M sodium succinate, 0.01 M potassium cyanide, 0.03 mM sodium 2,6-dichlorophenolindophenol, 0.3 mg/ml N-methyl phenozonium sulphate and enzyme. Enzyme activity was insignificant when succinate or N-methyl phenazonium sulphate was omitted.

One unit of enzyme activity is defined as the amount of enzyme which will cause a decrease of 0.01 per min at 600 nm in log (I_0/I) under the conditions specified by Hiatt (1961).

Catalase (EC 1.11.1.6) was determined by following the rate of

disappearance of H_2O_2 at 240 nm. The reaction was carried out at 25°C in 0.05 M potassium phosphate buffer (pH 7). The optical density of the substrate solution was initially 0.54. A change in log $(I_0/I)_{240}$ from 0.45 to 0.40 corresponded to the decomposition of 3.45 µmoles of H_2O_2 in 3 mls of solution.

Peroxidase (EC 1.11.1.7) activity was assayed by the method of Gregory (1966). The complete assay system contained: enzyme, 0.033 M sodium citrate buffer (pH 5.3), 0.02 ml of a saturated solution of benzidine in ethanol, 0.33 mM ascorbic acid and 0.066 M H_2O_2 in a total volume of 3 mls. A sudden development of blue color indicated the complete oxidation of ascorbic acid. The unit of activity is expressed as µmoles of ascorbic acid oxidized per min.

Glycollate oxidase (EC 1.1.3.1) was assayed by the method of Zelitch and Ochoa (1953), which utilizes the anaerobic reduction of 2,6-dichlorophenol indophenol. The complete reaction system contained: 0.2 M pyrophosphate buffer (pH 8.3), 0.1 mM dichlorophenol indophenol, 2 mM potassium cyanide, 0.2 mM NH_4OH, 0.07 mM FMN, 5 mM sodium glyoxylate and enzyme, in a total volume of 3 mls. After mixing, the solution in the cuvette was covered with a layer of toluene. One enzyme unit is defined as the amount which causes a decrease in log (I_0/I) of 0.01 per min at 620 nm.

Serine hydroxymethyltransferase (EC 2.1.2.1) was assayed by the method of Taylor and Weissbach (1965). This method is based on the principle that since the radioactive C-1 unit of 5,10-CH_2-H_4PteGlu readily equilibrates with carrier formaldehyde, it can be trapped as the 5,5-dimethyl-1,3-cyclohexadione adduct and measured. The complete reaction system contained: 0.1 µmole L-[3-^{14}C]serine (1 µCi/µmole),

0.1 µmole PALP, 30 µmoles potassium phosphate buffer (pH 8.3), 1.76 µmoles H_4PteGlu, 1 µmole 2-mercaptoethanol and enzyme in a total volume of 1 ml. Reactions were initiated by addition of substrate at 37°C and terminated 30 min later with 0.3 mls of 1 M sodium acetate (pH 4.5), 0.2 ml of 0.1 M formaldehyde and 0.3 ml of 0.4 M 5,5-dimethyl-1,3-cyclohexadione in 50% (v/v) ethanol. The HCHO-dimedon derivative was formed by heating for 5 min at 100°C, then extracted with 3 mls of toluene and counted.

N^5,N^{10}-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) was assayed by the method of Cossins *et al.* (1970). The complete assay mixture contained: 0.01 M potassium phosphate buffer (pH 7.5), 12.5 µmoles formaldehyde, 0.8 µmoles H_4PteGlu, 50 µmoles 2-mercaptoethanol, 1.8 µmoles NADP and enzyme in a final volume of 3 mls. The reaction was initiated by the addition of NADP and the change in absorbance at 340 nm was followed continuously.

N^{10}-formyltetrahydrofolate synthetase (EC 6.3.4.3) was assayed by the method of Hiatt (1965). When this enzyme was to be assayed, the extraction buffer included 10^{-4}M glutathione and 10^{-3}M 2-mercaptoethanol as additional constituents. The complete reaction system contained: 100 µmoles triethanolamine buffer (pH 8), 150 µmoles TRIS-formate (pH 8), 2.5 µmoles $MgCl_2$, 200 µmoles KCl, 4 µmoles H_4PteGlu, 2 µmoles ATP and enzyme in a total volume of 1 ml. After incubation for 20 min at 30°C the reaction was stopped by the addition of 2 mls of 0.36 N HCl. The absorbance at 355 nm was measured and the amount of 5,10-CH_2=H_4PteGlu formed was calculated from the extinction coefficient of 22×10^6 cm^2 per mole (Bertino, 1962). Because the activity of this enzyme was found to be variable between extractions, its activity was further

checked by microbiological assay of the N^{10}-formyl derivative formed. In such assays, the reaction system of Hiatt (1965) was used with the following modifications; instead of treating the reaction system with HCl, the pH was raised to 12 by addition of 5 M KOH followed by heating for 2 min at 60°C to convert 10-HCO-H_4PteGlu to 5-HCO-H_4PteGlu and oxidize H_4PteGlu remaining in the reaction system. The levels of 5-HCO-H_4PteGlu were then determined microbiologically using *P. cerevisiae* as described below under 'microbiological assay of pteroylglutamates'.

Electron microscopy of isolated particles. Fractions of the sucrose density gradient were embedded in agar and fixed at 2°C with 4% (v/v) Ladd glutaraldehyde in 0.01 M potassium phosphate buffer (pH 6.8) containing 50% (w/v) sucrose. The agar segments were then post-fixed in unbuffered 1% (w/v) OsO_4 for 30 min. The segments were dehydrated in an acetone series and embedded in epon. During dehydration, the particulate material was stained for 5 h in 70% (v/v) aqueous acetone containing 1% (w/v) uranyl nitrate. Sections were prepared on a Reichert Om U2 ultramicrotome using a Dupont diamond knife. Light gold sections were mounted on 200 mesh grids and stained with aqueous lead citrate for 3 min (Reynolds, 1963). The grids were then examined with a Phillips EM 200 electron microscope at 60 kV.

Respiratory-control determinations. Determinations of ADP/O ratios were performed with a YSI model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) with succinate and α-ketoglutarate as substrates (Chance and Williams, 1955, 1956).

The ratio, between a known amount of ADP (250 nmoles) added to the reaction mixture and the amount of oxygen utilized during esterification

of ADP to ATP, was calculated at 25°C from the cycle following the second addition of ADP. The complete reaction mixture as described by Malhotra and Spencer (1970) contained: 0.96 µmoles mannitol, 12 µmoles $MgCl_2$, 15 µmoles potassium phosphate, 150 mmoles TES (pH 7.2), 24 µmoles substrate and mitochondrial protein in a total volume of 3.2 mls. When α-ketoglutarate was the substrate 15 µmoles of malonate and 250 nmoles TPP were also added.

Microbiological assay of pteroylglutamates. Pteroylglutamates were assayed by the 'aseptic plus ascorbate' technique of Bakerman (1961), by using *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043), and *P. cerevisiae* (ATCC 8081). Standard reference curves were constructed by using PteGlu and 5-HCO-H_4PteGlu. The lactic acid produced after 72 h at 37°C was titrated and used as a measure of bacterial growth (Freed, 1966). Correlation of the titration values to standard reference curves, constructed with PteGlu and 5-HCO-H_4PteGlu, allowed determination of the concentration of derivatives before and after incubation of extracts with pea cotyledon γ-glutamyl carboxypeptidase (Roos and Cossins, 1971).

Chromatography of pteroylglutamate derivatives. Column chromatography of pteroylglutamate derivatives was performed on DEAE-cellulose columns (20 cm x 1.8 cm) by using a continuous concentration gradient of potassium phosphate buffer (pH 6) in the presence of ascorbate (Roos *et al.*, 1968; Roos and Cossins, 1971). Pteroylglutamate derivatives were identified using the basic criteria of differential growth response described earlier (Sengupta and Cossins, 1971; Roos and Cossins, 1971; Cossins and Shah, 1971). Generally *L. casei* requires low concentrations of PteGlu or its derivatives for growth, whereas *S. faecalis* requires a higher concentration of PteGlu,

H_4PteGlu, formyl substituted derivatives of H_4PteGlu, or pteroic acid to sustain growth. *P. cerevisiae* has a specific requirement for formyl derivatives of H_4PteGlu and will not grow on methyl derivatives. The degree of relative growth response on different derivatives for either organism is not equivalent and has been summarized in part by Rohringer *et al.* (1969).

Solubilization of mitochondrial pteroylglutamates. After fractionation of the sucrose density gradient, mitochondrial fractions were immediately subjected to various solubilization treatments. These included (a) sonication at full amplification with a Model BPO Fisher Ultrasonic Generator (Blackstone Ultrasonics Inc., Sheffield, Pa., U.S.A.) for 1 min at 4°C; (b) three fold dilution with 1% (w/v) potassium ascorbate (pH 6), and incubation with 2% (w/v) sodium deoxycholate for 30 min at 4°C; (c) three fold dilution with 1% (w/v) potassium ascorbate (pH 6); (d) sonication for 1 min followed by treatment with 2% (w/v) deoxycholate for 30 min at 4°C; and (e) treatment with 2% (w/v) deoxycholate without dilution. After such treatments, the fraction was placed over a concentrated sucrose layer and centrifuged in a Spinco SW 40 rotor at 30,000 rev/min (119,000*g* at $R_{av.}$) for 20 min. Pteroylglutamate content of the sedimented and soluble fractions, from this step, was then assayed microbiologically with *L. casei* and *P. cerevisiae*.

Carbon-14 feeding experiments. One hundred seeds, selected after the first 30 min of imbibition, were allowed to imbibe 20 µCi of [*methyl*-^{14}C]-5-CH_3-H_4PteGlu acid (61 µCi/µmole), or 25 µCi of [2-^{14}C]-PteGlu (55.3 µCi/µmole). After uptake of the isotope the seeds were allowed to complete imbibition in water as before and germinated in

petri dishes for 88 h on moist filter paper in darkness at 25°C.

Counting of radioactive samples. Radioactive samples were counted in a liquid scintillation counter (Nuclear-Chicago Corporation, Unilux II model). Aliquots (50-200 µl) of the radioactive solutions were counted in 15 mls of fluor containing 6.5 g 2.5-diphenyloxazol (PPO) and 0.65 g 1,4-*bis*[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl POPOP)/l of dioxane:anisole:dimethoxyethane (6:1:1 by volume). The counting efficiency was 78% as determined by calibration with a [^{14}C]toluene internal standard. All counts were corrected for background (22 cpm) and were only regarded as significant if at least three times this level.

Radioactive samples absorbed in hyamine hydroxide were counted in 15 mls of Bray's solution (Bray, 1960) at similar efficiencies.

Biosynthesis of pteroylglutamates 'in vitro'. Aliquots of the mitochondrial fraction (approx. 1 mg protein) were incubated at 35°C for 20 min with 4 nmoles H_4PteGlu under buffered conditions. The specific quantities of substrates and adenine nucleotides added to the reaction system in the various experiments and their controls are given in the appropriate tables. Reduced FAD was generated *in situ* by incubation with diaphorase (lipoic dehydrogenase, Sigma Chemical Company) and NADH or NADPH (Figure 1) for 10 min at 35°C before addition of mitochondrial protein. Diaphorase activity was routinely verified by following the oxidation of NADH at 340 nm (Figure 2). Decreases in log $(I_0/I)_{340}$ were shown to have absolute requirements for diaphorase and FAD.

The biosynthesis of pteroylglutamates was terminated by boiling the reaction system for 2 min after addition of 1 ml potassium

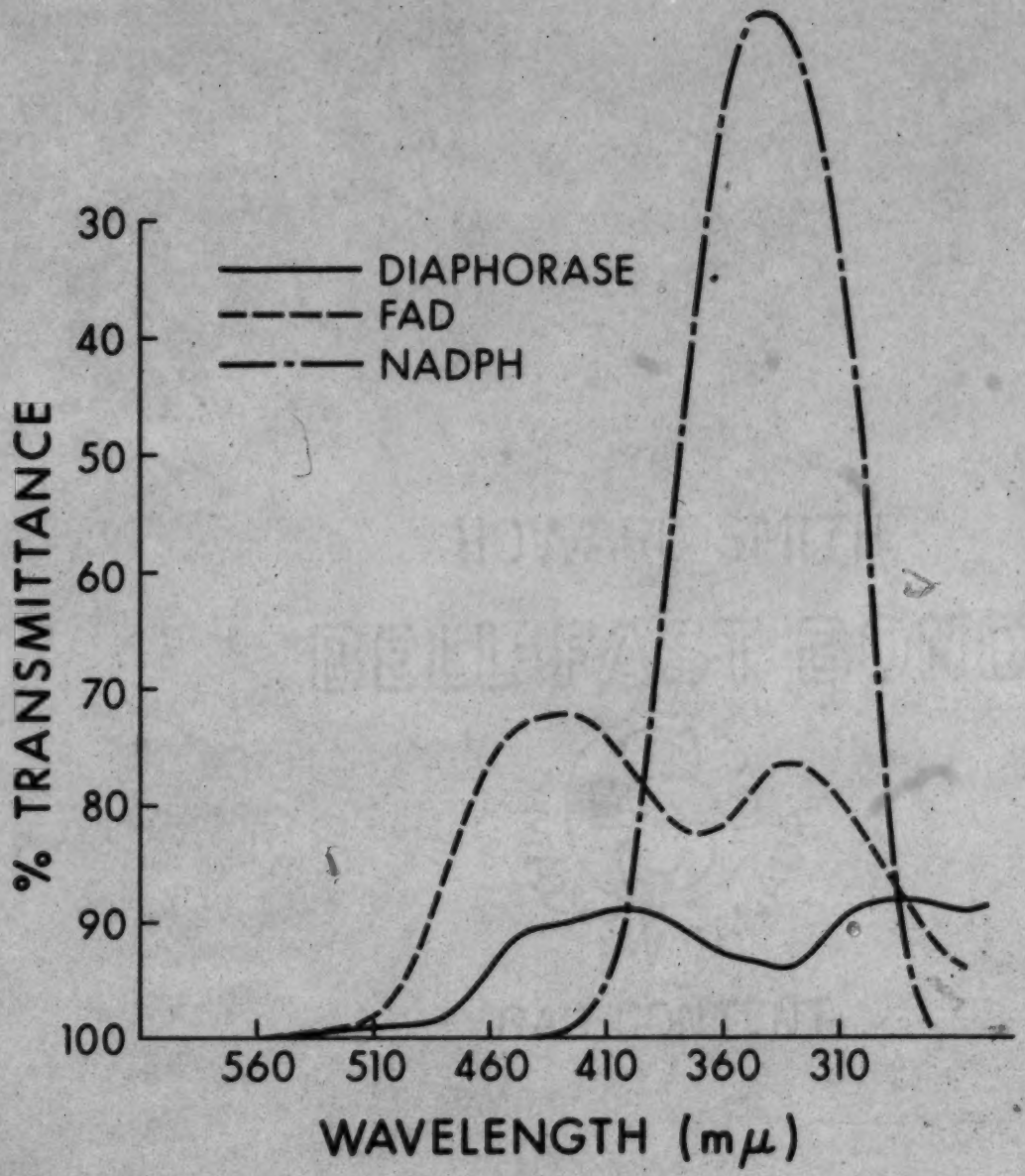


FIGURE 1. Spectrophotometric scan of the reaction components utilized for the *in vitro* synthesis of $FADH_2$. The components were scanned in 40 mM potassium phosphate buffer (pH 7.4), containing 8 nmoles H_4PteGlu per ml.

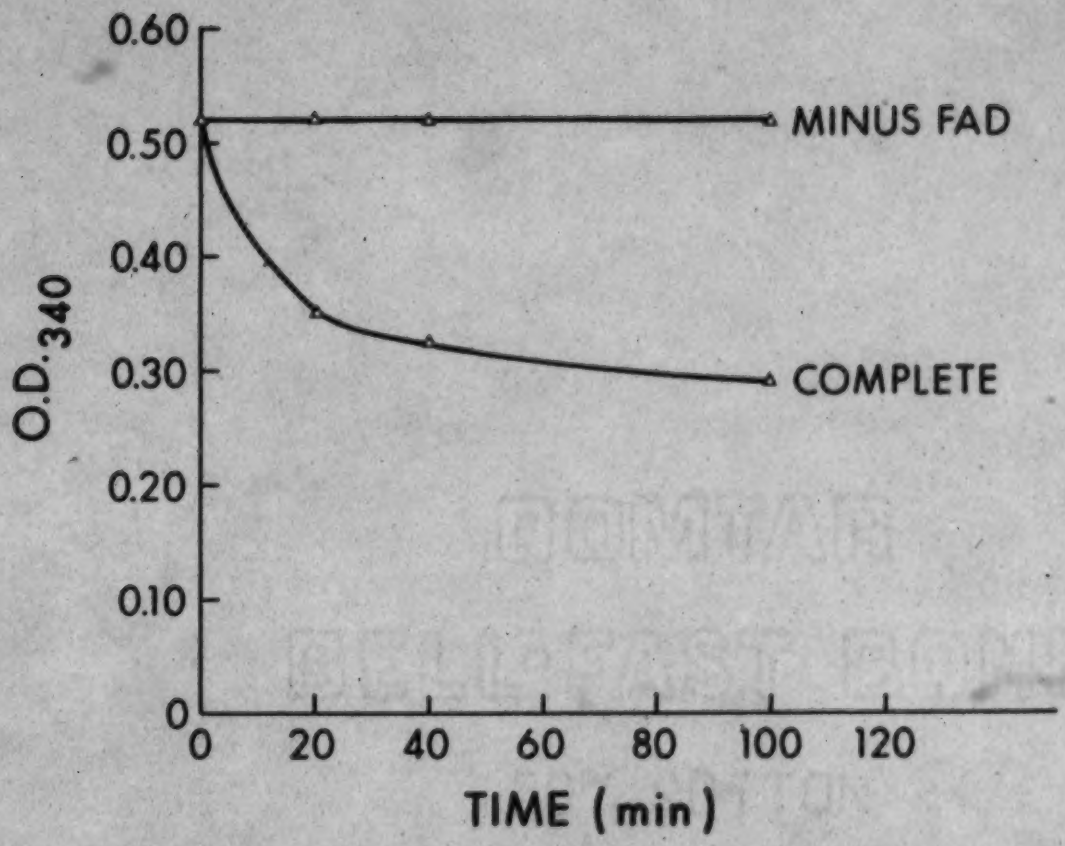


FIGURE 2. Oxidation of NADH by diaphorase. The complete reaction mixture contained: 0.3 units lipoamide dehydrogenase, 24 μmoles FAD, 24 μmoles NADH, 4 nmoles H_4PteGlu and 40 μmoles potassium phosphate buffer (pH 7.4) in a total volume of 3 mls. The FAD dependent oxidation of NADH and concomitant reduction of FAD to $FADH_2$ were monitored by following the decrease in optical density at 340nm .

ascorbate (1.2% w/v, pH 6). Aliquots containing approximately 1 µg of pteroylglutamates, were then chromatographed on DEAE-cellulose. Fractions from the columns were assayed for ^{14}C and the presence of pteroylglutamate derivatives was verified by *L. casei* growth response.

Chromatography of labelled free amino acids. Amino acids formed from ^{14}C-labelled substrates were separated using a Beckman Automatic Amino Acid Analyzer Model 121 (Beckman Instruments Inc., Palo Alto, California, U.S.A.) equipped for stream division of the column effluent (Clandinin and Cossins, 1972). Before such analysis, a portion of the reaction system was passed through a column of Dowex 50W-X8 (H^+ form) 100-200 mesh (BioRad Laboratories, Richmond, California, U.S.A.). The column was then washed with deionized water and the amino acids eluted with 2 N HCl. After removal of the HCl *in vacuo* on a Buchler flash-evaporator, the amino acids were dissolved in 0.2 M citrate buffer (pH 2.2) and subjected to chromatography. Acidic and neutral amino acids were eluted from a 54 cm bed of Beckman Spinco P.A. 28 resin using a pH 3.15 and 4.22, 0.2 N citrate buffer system. Basic amino acids were eluted from a 12 cm resin bed of Beckman Spinco P.A. 35 using 0.38 N citrate buffer (pH 5.25). The reagent buffers were adjusted to their respective pH values at 23°C, and elution was carried out at 53°C, at a flow rate of 70 ml/h. Radioactive peaks were identified by co-chromatography with internal standards. Radioactive amino acid peaks were further identified by thin layer chromatography on 20 x 20 cm Silica Gel GF plates (Mandel Scientific Co., Montreal, Quebec, Canada) using phenol:water (3:1 v/v) and propanol:water (7:3 v/v) as solvent systems (Table 2, Plates 1 and 2).

TABLE 2. *Summary of Rf values for various amino acids subjected to thin layer chromatography*

Solvent	1	2	3
Alanine	0.834	0.634	0.427
Glycine	0.375	0.594	0.406
Histidine	1.0	0.360	0.280
Methionine	0.855	0.760	0.566
Glutamate	0.898	0.691	0.586
Serine	0.757	0.582	0.433
Methionine sulphoxide	0.823	0.417 & 0.748	0.300
α-methylmethionine	0.852	0.771	0.745
Methionine sulphone	0.497	0.611	0.473
Homocystine	0.342	0.605	0.326
Homoserine	0.897	0.617	0.446
Threonine	0.899	0.622	0.466
Methyl serine	0.673	0.727	0.475
Ethyl serine	0.714	0.784	0.550
Homocysteine	0.952 & 0.327	0.786 & 0.726	0.645
Homocysteine-thiolactone	0.952	0.786	0.645
Aspartate	0.193	0.697	0.766
Cysteine	0.243 & 0.460	0.697 & 0.469	0.660 & 0.787 & 0.823

The compounds were dissolved in water wherever possible and the amount applied to the chromatogram was 0.01 - 0.1 μmole.

Solvent 1 = phenol:H_2O (3:1 by volume)

Solvent 2 = *n*-butanol:methylethyl ketone; formic acid:H_2O (8:6:3:3 by volume)

Solvent 3 = *n*-propanol:water (7:3 by volume)

PLATE 1. An example of the separation of amino acids achieved with phenol:H_2O (3:1 v/v).

The TLC plates were sprayed with 0.5% w/v ninhydrin in acetone then warmed briefly at 90°C and subsequently sprayed with 0.5% nickelous sulphate to stabilize the colored spots.

The amino acids viewed from left to right are:

alanine, serine, glycine, histidine, methionine, methyl serine, methionine sulphone, methionine sulphoxide, homoserine and threonine.

PLATE 2. An example of the separation of amino acids achieved with *n*-propanol:H_2O (7:3 v/v).

The line of origin is marked and the solvent front was 20 cm from the origin. The arrow indicates the direction of solvent travel.

The amino acids viewed from left to right are:

alanine, glycine, histidine, methionine, glutamate, serine, methionine sulphoxide, methionine sulphone, homoserine, threonine.

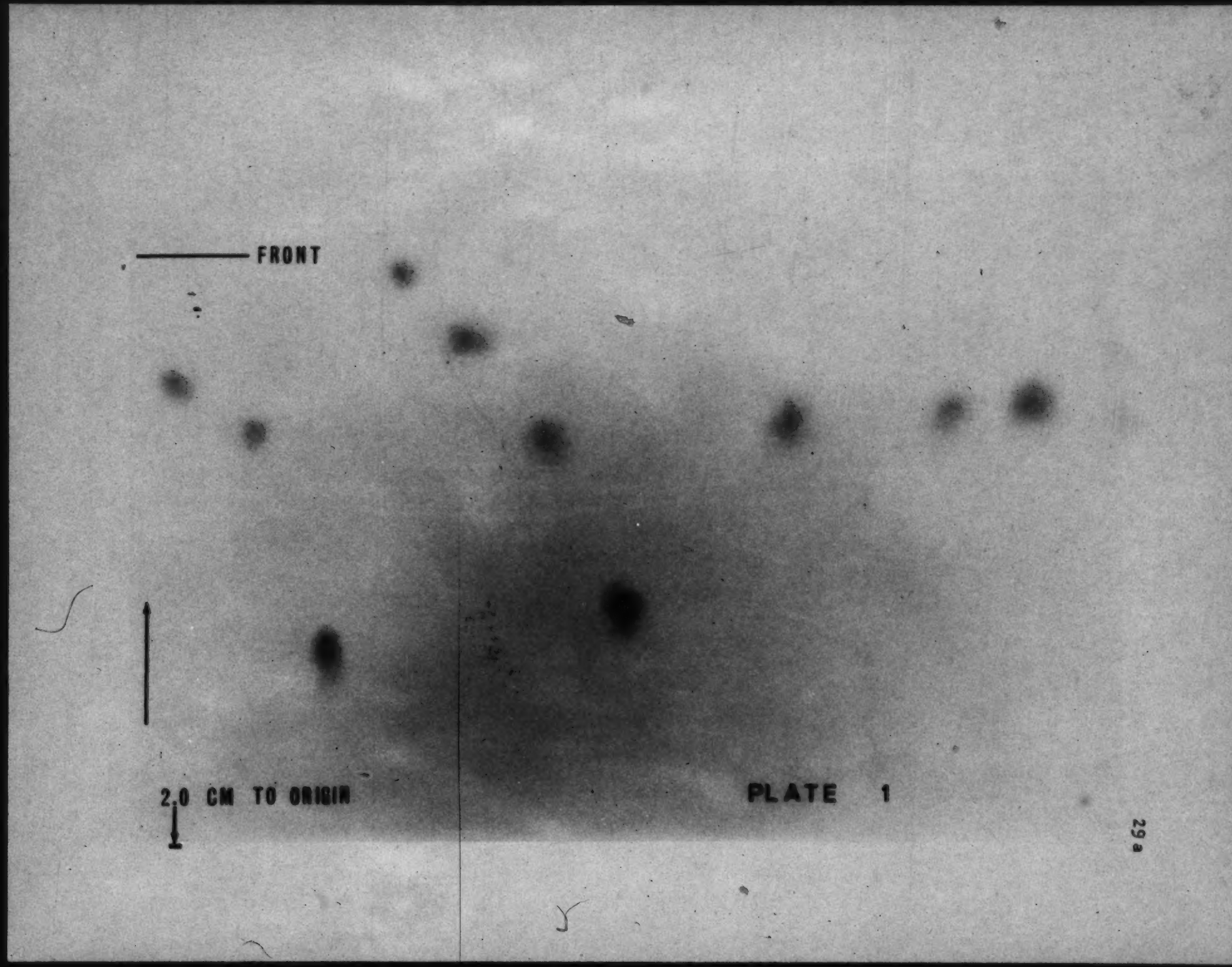
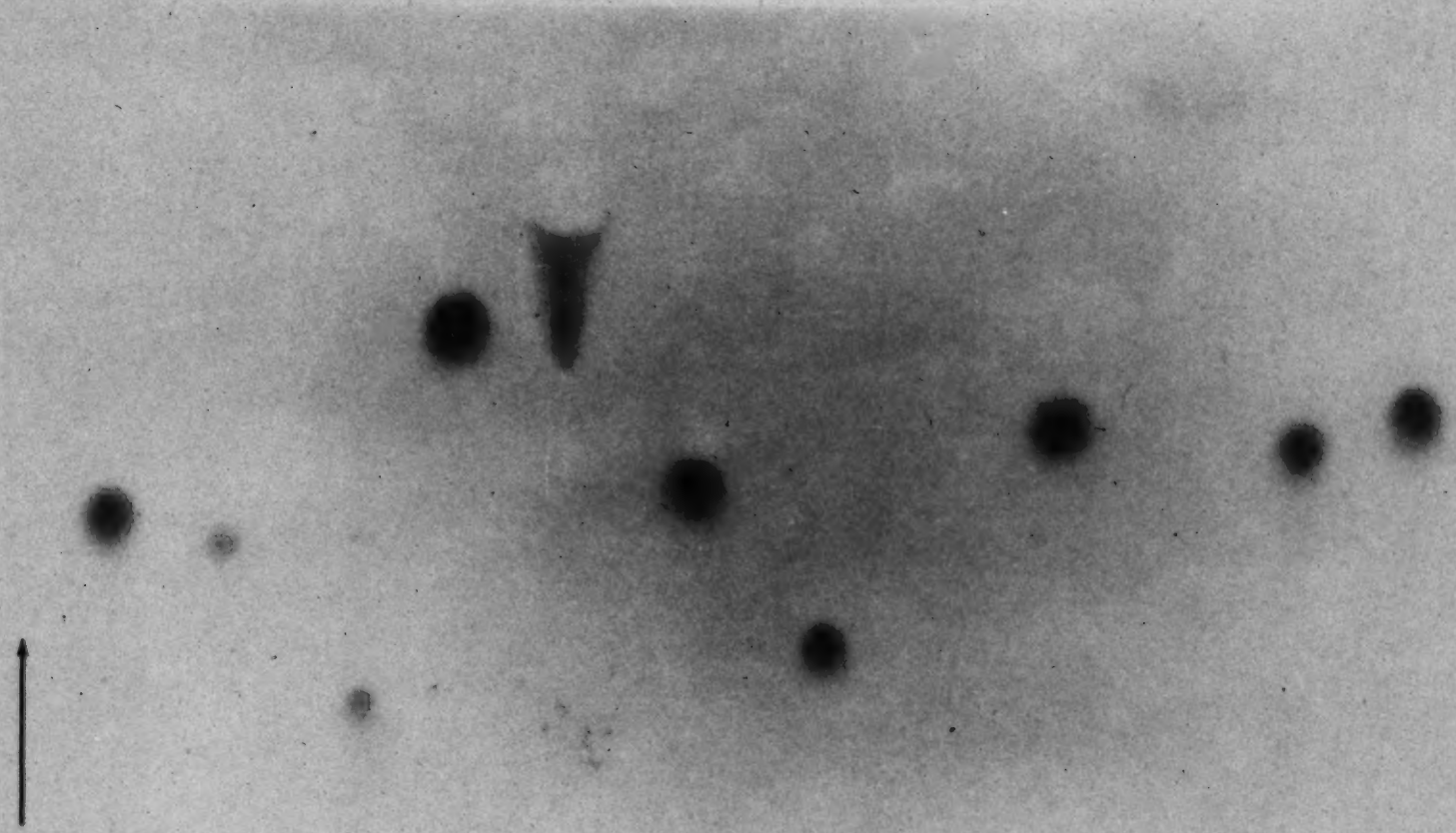


PLATE 1



ORIGIN

PLATE. 2

Assay of glycine decarboxylase and the bicarbonate exchange reaction. The exchange of bicarbonate with the carboxyl carbon of [1-^{14}C]glycine was assayed by essentially the system of Sato *et al.* (1969) with the following modifications. Reactions were carried out aerobically, (20% O_2) except in experiments where the effects of O_2 tension were tested. The reaction components, in stoppered Warburg manometric flasks, were incubated at 37°C for 30 min. The complete reaction system (2 mls) consisted of: 10 µmoles [1-^{14}C]glycine (0.05 µCi/µmole), 1.5 µmoles PALP, 10 µmoles dithiothreitol, 100 µmoles Tris-HCl buffer (pH 7.8), 60 µmoles sodium bicarbonate, 1 mmole sucrose and aliquots of mitochondrial fraction.

The reaction was stopped and the $^{14}CO_2$ liberated from the solution by addition of 0.3 mls of 4N H_2SO_4 tipped from the side arm of the flask. The CO_2 liberated was absorbed by 0.4 mls of 50% (v/v in methanol) hyamine hydroxide previously placed in the center well of the flask. CO_2 was absorbed for 45 min while the flask was agitated at 50°C in a shaker bath. The contents of the center well were then counted in 15 mls of Bray's solution.

Glycine decarboxylase assay was carried out under the same conditions, except that 1.25 µmoles of NAD^+ and 2 nmoles of H_4PteGlu were added while bicarbonate was omitted.

Variations of these two reaction systems and their controls are specificially described in their respective tables and figures.

Extraction of 5-CH_3-H_4PteGlu methyltransferase catalyzing the biosynthesis of methionine. The mitochondrial fraction purified as previously described was subjected to ammonium sulphate fractionation as described by Dodd and Cossins (1970). The mitochondrial fraction,

contained in a volume of 10 mls, was raised to 20% of saturation by addition of solid $(NH_4)_2SO_4$ at 4°C. After continuous stirring for 30 min, the precipitated protein was removed by centrifugation (10,000*g* for 10 min). The supernatant was then raised to 60% of saturation and stirred for 30 min before removal of the precipitated protein. This 20-60% protein fraction was dissolved in 10 mls of 0.05 M potassium phosphate buffer (pH 6.9) containing 5 mM 2-mercaptoethanol and passed through a column of Sephadex G-25 (1 x 5 cm). The resulting solution was used for assays of methyltransferase activity.

Assay of methyltransferase activity. The reaction systems used to assay the homocysteine-dependent methyltransferases have been previously described (Dodd and Cossins, 1970). The standard reaction mixture in a total volume of 0.5 ml contained: enzyme fraction (1 mg protein), 1 µmole L-homocysteine, 1.6 mµmole [*methyl*-^{14}C]-5-CH_3-H_4PteGlu (2.0×10^5 dpm) or SAM-[*methyl*-^{14}C] (1.82×10^5 dpm) and 50 µmoles potassium phosphate buffer (pH 6.9). After incubation at 30°C for 60 min, the reaction was terminated by rapid cooling in an ice bath. An aliquot (0.1 ml) of the chilled reaction mixture was placed on a column (0.5 x 2.5 cm) of Dowex AG1-X10 (Cl^- form) resin when 5-CH_3-H_4PteGlu was the methyl donor or when labelled SAM was the methyl donor the aliquot was applied to a similar column containing Dowex 50W-X8 resin in Li^+ form (Abramson and Shap[illegible]). The [*methyl*-^{14}C]methionine synthesized was eluted with five washings, each of 0.2 ml distilled water, and collected directly in a scintillation vial for counting.

One unit of enzyme activity is defined as the amount of enzyme producing 1 µmole of methionine in 1 min at 30°C under the reaction conditions described.

Assay of homoserine transacetylase or homoserine transsuccinylase. The principle of this assay method is based upon the method of Nagai and Kerr (1971), and the fact that O-acetylhomoserine (or O-succinylhomoserine) formed in the following reaction is converted by mild alkaline treatment to N-acetylhomoserine. N-acetylhomoserine can then be readily separated since it is not retained by Dowex 50W-X8 in H^+ form.

3. L-homoserine + S-acetyl-CoA → O-acetyl-L-homoserine + CoA

4. L-homoserine + S-succinyl-CoA → O-succinyl-L-homoserine + CoA

The complete assay system in a total volume of 0.5 mls contained: 50 µmoles potassium phosphate buffer (pH 7.5), 0.5 µmole S-acetyl-CoA (or S-succinyl-CoA), 50 nmoles [U-^{14}C]-L-homoserine (1 µCi/µmole) and 3 to 4 mg protein of the mitochondrial fraction. The reaction mixtures were incubated at 37°C for 30 min then stopped by adding 50 µl of 1.5 M trichloroacetic acid. The precipitate was removed by centrifugation and 250 µl of the supernatant was transferred to another tube containing 100 µl of 1 M KOH. The mixture was then placed in a boiling water bath for 1 min, then cooled and 150 µl were transferred to a column of Dowex 50W-X8, H^+ form, (0.5 x 5 cm). The column was washed with five 0.5 ml aliquots of distilled water directly into a scintillation vial for direct counting of the N-acetylhomoserine eluted.

Synthesis of SAM. The *in vitro* synthesis of SAM by the same mitochondrial extracts was conducted in a reaction system of 2 mls containing: 8 µmoles [*methyl*-^{14}C]-L-methionine (0.125 µCi/µmole), 10 µmoles ATP, 5 µmoles 2-mercaptoethanol, 50 µmoles $MgCl_2$, 100 µmoles potassium phosphate buffer (pH 6.9), and 2 mg protein prepared as described. The reaction mixtures were incubated for 60 min at 30°C,

chilled, and the labelled SAM synthesized was separated by the column chromatographic method of Shapiro and Ehninger (1966). The method was modified to include the use of HCl instead of H_2SO_4 as the eluting acid, thus enabling the removal of acid *in vacuo* permitting liquid scintillation counting of the aqueous samples without the substantial quenching problems of acidic extracts.

Synthesis of SMM. The *in vitro* synthesis of SMM by the same mitochondrial extracts utilized for other transmethylase studies, was attempted in a reaction system of 1 ml containing: mitochondrial extract (2 mg protein), 8 µmoles L-methionine, 0.05 µmole SAM-[*methyl*-^{14}C] (52 µCi/µmole), 5 µmoles 2-mercaptoethanol and 100 µmoles potassium phosphate buffer (pH 6.9). The reaction mixtures were incubated for 1.5 h at 30°C. Reaction products were identified by TLC and autoradiography using a solvent system of *n*-butanol:acetic acid:H_2O (12:3:3) and silica gel G TLC plates (Dodd, 1969).

Preparation of β-cystathionase and cystationine-γ-synthase. The mitochondrial fraction isolated as previously described was diluted two fold with 10 mM potassium phosphate buffer (pH 7.3), sonicated for 45 sec and then centrifuged at 14,000*g* for 40 min. The 14,000*g* supernatant was raised to 45% of saturation with solid $(NH_4)_2SO_4$ and then stirred for 30 min. The resulting precipitate was collected by centrifugation (10,000*g* for 15 min) and dissolved to 4 mls total volume in 10 mM potassium phosphate buffer (pH 7.3). This solution termed the 0-45% fraction was passed through a column of Sephadex G-25 (1 x 5 cm) and used directly in assays of β-cystathionase and cystathionine-γ-synthase.

Assay of β-cystathionase.

5. L-cystathionine + H_2O → L-homocysteine + pyruvic acid + NH_3

The enzyme activity was determined by a reaction sequence described by Guggenheim (1971) and similar to the system utilized for assays of cystathionine-γ-synthase. The production of pyruvate by β-cystathionase was coupled to an excess of lactic dehydrogenase in the presence of NADH. Measurements of homocysteine production with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) were not found to yield satisfactory reaction rates and consequently the following assay system was utilized. The complete reaction mixture in a final volume of 1 ml contained: 50 μmoles potassium pyrophosphate buffer (pH 8.2), 0.1 μmole pyridoxal-5'-phosphate, 4 μmoles L-cystathionine and enzyme fraction (400 μgm of protein). Control reaction systems contained no L-cystathionine. The reaction tubes were incubated at 37°C for 10 min then the reaction was terminated by addition of 50 μl of 1.5 M trichloroacetic acid. After centrifugation, aliquots of the reaction systems were assayed for pyruvate using lactic dehydrogenase utilizing the same system described for cystathionine-γ-synthase.

Assay of cystathionine-γ-synthase. The assay procedure used was similar to that described by Kaplan and Guggenheim (1971) for reaction 7 (see below), known to be catalyzed by cystathionine-γ-synthase of *S. typhimurium.*

6. O-Succinyl-L-homoserine + L-cysteine → L-cystathionine + succinic acid

7. O-Succinyl-L-homoserine + H_2O → α-ketobutyrate + NH_3 + succinic acid

The α-ketobutyrate formed in reaction 7 was measured with NADH and an

excess of beef heart lactic dehydrogenase. A two step assay procedure was utilized because of the instability of NADH in the extracts. Preliminary trials using inhibitors such as; rotenone, amytal or antimycin A up to concentrations of 1 mM, known to inhibit NADH oxidase activity during electron transport, were not found to stabilize NADH in the presence of the extracts used in this study.

The first step of this assay procedure utilized a reaction mixture (0.5 ml) containing: 50 µmoles potassium pyrophosphate buffer (pH 8.2), 0.1 µmole pyridoxal-5'-phosphate, 2.5 µmoles O-succinyl-DL-homoserine and 2 mg enzyme protein. Control mixtures contained no O-succinyl-homoserine.

The reaction mixtures were incubated at 37°C for 10 min then stopped by adding 50 µl of 1.5 M trichloroacetic acid. After centrifugation aliquots of the supernatant solution were added to cuvettes containing, in a final volume of 1 ml: 100 µmoles potassium phosphate, (pH 7.3), and 0.15 µmole NADH. After ascertaining that the pH was above neutrality, the E_{340} was measured and a large excess of cystalline lactic dehydrogenase (20 µgm) was added. After the E_{340} had ceased to decline the optical density was recorded. The amount of α-ketobutyrate in the aliquot was calculated from the difference between the two readings using a molar absorbance of 6200.

RESULTS

Fractionation of Organelles

After isopycnic non-linear density gradient centrifugation of the particulate fraction, separate bands were detected, which on the basis of their enzyme complements, included mitochondria, in fraction 5, and peroxisome-like bodies in fractions 3 and 4 (Table 3). Fraction 5 was characterized as containing mainly intact mitochondria by the presence of comparatively high levels of fumarase and succinic dehydrogenase (61.7% and 61.5% respectively, of the total enzyme activity present in the crude particulate fraction). Fraction 5 also contained low levels of peroxidase and glycollate oxidase (9.5% and 22.8%, respectively).

Electron microscopy revealed that fraction 5 contained intact mitochondria with some contamination by mitochondrial fragments. No microbodies could be detected. When ADP/O ratios were determined with succinate and α-ketoglutarate as substrates, the mitochondrial fraction was found to have respiratory control values of 2.5 and 4 respectively, indicating that some degree of integrity existed in the organelles of this fraction. It should be noted that the levels of inorganic phosphate present in the isolating medium would have substantial effects on the ADP/O ratios obtained, likely inhibiting maximal respiratory activity and consequently optimum ADP/O ratios.

Localization of pteroylglutamate derivatives and related enzymes

Correlation was found between the distributions of serine hydroxymethyltransferase and other enzymes characteristic of mitochondria (Table 3). Although the former enzyme and 10-HCO-H_4PteGlu synthetase

TABLE 3. *Distribution of particulate enzymes within fractions of the sucrose density gradient*

Enzyme	Fraction No. 1	2	3	4	5	6	7
Fumarase							
Activity	0.313	0.95	0.50	1.51	5.28	*n.d.*	*n.d.*
Distribution (%)	3.66	11.1	5.88	17.7	61.7	--	--
Succinic Dehydrogenase							
Activity	*n.d.*	*n.d.*	*n.d.*	1.16	2.15	*n.d.*	0.185
Distribution (%)	--	--	--	33.2	61.5	--	5.29
Peroxidase							
Activity	0.052	0.089	0.055	0.188	0.041	0.007	*n.d.*
Distribution (%)	12.0	20.6	12.7	41.9	9.51	1.56	--
Catalase							
Activity	0.192	0.947	2.92	2.06	2.06	2.18	0.097
Distribution (%)	2.26	11.2	34.4	24.2	24.2	2.57	1.15
Glycollate Oxidase							
Activity	0.067	0.151	0.235	4.97	2.04	0.138	1.38
Distribution (%)	<1	1.68	2.62	55.5	22.8	1.81	15.4
Serinehydroxymethyltransferase							
pmole HCHO formed	0.339	1.0	0.831	2.07	15.0	2.37	0.349
Distribution (%)	1.40	4.20	3.40	10.4	62.4	9.86	1.50
10-HCO-H_4PteGlu synthetase							
nmol 10-HCO-H_4PteGlu formed	*n.d.*	*n.d.*	*n.d.*	*n.d.*	1.81	*n.d.*	*n.d.*
Distribution (%)							

n.d. - not detectable.

The data represent mean values of at least three separate extractions. Enzyme activities are expressed in units/g fresh weight of cotyledons.

were associated with the mitochondrial fraction, 5,10-CH_2-H_4PteGlu dehydrogenase was not detected by the spectrophotometric method. The presence of the latter enzyme could not be entirely ruled out however, as substantial mitochondrial NADH oxidase was present in fraction 5. The activity of 10-HCO-H_4PteGlu synthetase, although apparently restricted to the mitochondrial fraction, may be to some extent also present in other particulate fractions as difficulty was also encountered in assay of this enzyme by the spectrophotometric method.

Preliminary microbiological assays of total pteroylglutamates illustrated that relatively high levels occurred in the 27,000*g* pellets obtained from 4-day-old cotyledons. Further examination of these by sucrose density gradient centrifugation (Table 4) revealed that 63% of the *L. casei* growth response and 54% of that given by *P. cerevisiae* was associated with the mitochondrial fraction. The difference in total levels given by these two organisms indicates that methyl and formyl derivatives of H_4PteGlu with possibly different degrees of conjugation were present in this fraction. The presence of pteroylglutamates in the denser fractions of the sucrose gradient suggests that these compounds may also be associated with mitochondrial fragments.

The total pteroylglutamate levels of the mitochondrial fraction as measured by *L. casei* without pre-treatment of the extracts with conjugase, amounted to approximately 7 to 10% of the total pteroylglutamate content of 4-day-old cotyledons (Table 5). Repeated washing of the mitochondrial fraction reduced this figure by as much as 50%, indicating that these derivatives are readily leached out of this organelle.

TABLE 4. *Distribution of pteroylglutamate levels in fractions of the sucrose density gradient*

Fraction number	*L. casei*		*P. cerevisiae*	
	ng	Distribution (%)	g	Distribution (%)
1	0.806	2.33	0.493	2.79
2	0.940	2.72	0.806	4.57
3	0.672	1.94	1.075	6.10
4	2.149	6.22	1.746	9.91
5*	21.940	63.47	9.433	53.52
6	4.702	13.60	3.134	17.78
7	3.358	9.72	0.940	5.33
Total	34.567	100	17.627	100

Pteroylglutamate levels are expressed in ng PteGlu for *L. casei* and ng 5-HCO-H_4PteGlu for *P. cerevisiae*/g fresh weight of cotyledons extracted.

* Incubation of fraction 5 with pea cotyledon γ-glutamyl carboxypeptidase resulted in an increase in the growth response of *L. casei* and *P. cerevisiae* of 51 and 47 percent, respectively.

TABLE 5. *Levels of pteroylglutamates in the mitochondrial and supernatant fractions of pea cotyledons*

	Pteroylglutamates* ng/g fresh weight	
	Before conjugase	After conjugase
Mitochondrial fraction	21.9	30.6
Supernatant fraction	220	458

*Pteroylglutamate content was assayed with *L. casei* and is expressed as ng equivalents of PteGlu.

Release of pteroylglutamates from mitochondria by solubilization treatments

Solubilization treatments, summarized in Table 6, confirmed that the mitochondrial fraction contained a pool of pteroylglutamates that are bound more tightly than can be explained solely by adsorption or diffusion of these compounds during extraction of the mitochondria. Eighty-five percent of the total pteroylglutamates, as measured by *L. casei*, was retained by the mitochondrial debris after treatment by osmotic shock suggesting that the derivatives were in large part membrane-bound. This contention was supported by the observation that after sonication, 15% of the total pteroylglutamate content was retained by the mitochondrial debris but this figure was reduced after treatment with deoxycholate. With one exception, analogous results were obtained with *P. cerevisiae* after these treatments. In most treatments, release of pteroylglutamates to the supernatant was higher when the levels were determined with *P. cerevisiae*, indicating that formylated derivatives may be less tightly bound than methylated and/or

TABLE 6. *Solubilization of pteroylglutamate derivatives from isolated mitochondria*

Treatment	*L. casei* Particulate fraction %	*L. casei* Supernatant fraction %	*P. cerevisiae* Particulate fraction %	*P. cerevisiae* Supernatant fraction %
Osmotic shock	84	16	21	79
Osmotic shock and 2% deoxycholate	<1	99	<1	99
Sonication	15	85	2.6	97.4
Sonication and 2% deoxycholate	<1	99	<1	99
2% deoxycholate	1	99	3.2	96.8

conjugated derivatives.

Chromatography of mitochondrial pteroylglutamates

Figures 3A and 3B are typical elution patterns of the derivatives present in the mitochondrial fraction. The first major peaks (Figure 3B, peaks *a*, *c*, and *e*) gave growth promoting properties typical of formyl derivatives and occupied positions in the elution sequence corresponding to authentic 10-HCO-H_4PteGlu, 5-HCO-H_4PteGlu and 5-HCO-H_4PteGlu$_2$ respectively (Roos and Cossins, 1971). The large *L. casei* peak (Figure 3B, peak *d*) coincided with authentic 5-CH_3-H_4PteGlu. A small shoulder at fractions 76-78 (Figure 3B) may represent H_4PteGlu. Other derivatives present co-chromatographed with standard derivatives (Roos and Cossins, 1971) and were: Peak *b*, 10-HCO-H_4PteGlu, and peak *f*, 5-CH_3-H_4PteGlu$_2$. In addition, peaks *g* to *j*, identified in earlier work (Roos and Cossins, 1971) as conjugated derivatives were also present.

Roos and Cossins (1971) have suggested that PteGlu may be an intermediate in the synthesis of more highly reduced compounds such as 5-CH_3-H_4PteGlu in pea cotyledons. In order to examine this possibility and to determine whether the mitochondrial pool of pteroylglutamates would be derived from such a precursor, [2-^{14}C]PteGlu and [*methyl*-^{14}C]-5-CH_3-H_4PteGlu were supplied during imbibition. Labelled derivatives were detected in the mitochondrial pool after such feeding (Figure 4). [*Methyl*-^{14}C]-5-CH_3-H_4PteGlu labelled 10-HCO-H_4PteGlu, 5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu more readily than in similar experiments which involved [2-^{14}C]PteGlu. Labelled PteGlu was incorporated into most of the mitochondrial pteroylglutamates but the specific activity of these was too low to permit clear resolution of individual derivatives. This

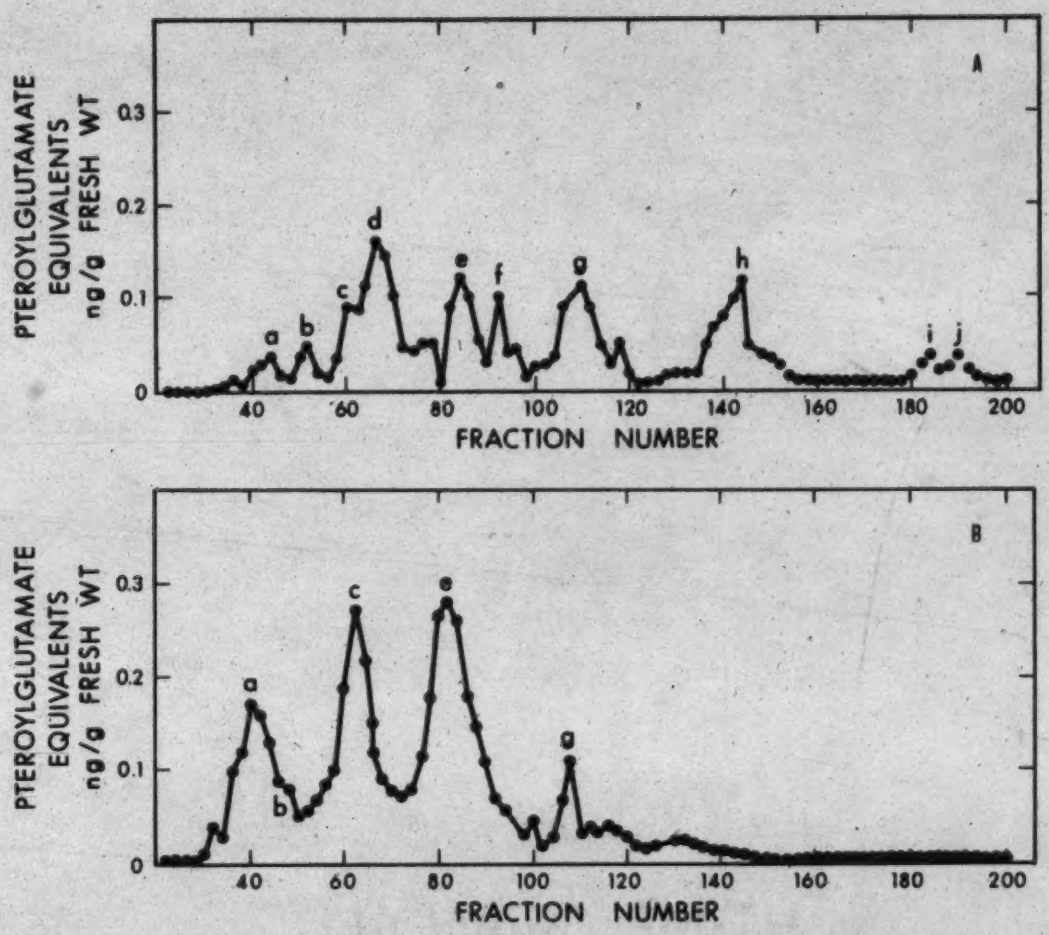


FIGURE 3. Chromatography of pteroylglutamate derivatives from isolated mitochondria. After DEAE-cellulose chromatography the fractions were assayed for pteroylglutamates using *L. casei* (A) and *P. cerevisiae* (B). The derivatives shown are:

a, 10-HCO-H_4PteGlu; *b*, 10-HCO-H_4PteGlu$_2$;
c, 5-HCO-H_4PteGlu; *d*, 5-CH_3-H_4PteGlu; *e*, 5-HCO-H_4PteGlu$_2$;
f, *g*, *h*, *i*, *j*, unidentified conjugated derivatives.

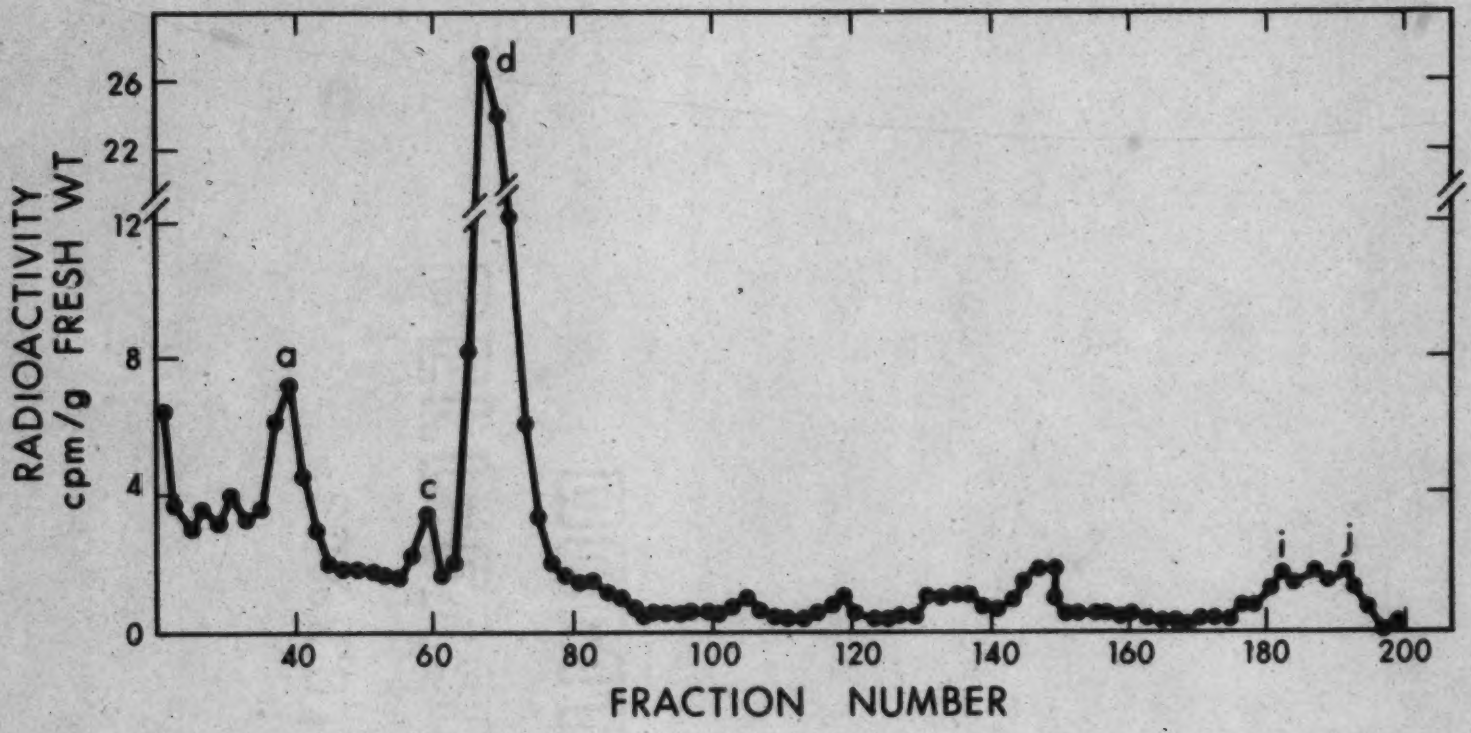


FIGURE 4. Separation of labelled mitochondrial pteroylglutamate derivatives by column chromatography on DEAE-cellulose, after [*methyl*-^{14}C]-5-CH_3-H_4PteGlu was supplied to the germinating seeds. The identified peaks shown are:

a, 10-HCO-H_4PteGlu; *c*, 5-HCO-H_4PteGlu; *d*, 5-CH_3-H_4PteGlu; *i* and *j*, unidentified derivatives.

finding might be related to the dramatic synthesis of pteroylglutamates which occurs in this tissue (Roos and Cossins, 1971), thus diluting the specific activity of the substrate incorporated.

The presence of pteroylglutamate derivatives and the occurrence of related enzymes in pea mitochondria suggests that these derivatives are interconvertible and may have metabolic significance in the synthesis and metabolism of related free amino acids, which could also be detected in the isolated mitochondria (Table 7). The presence of serine hydroxymethyltransferase and 10-HCO-H_4PteGlu synthetase suggests that one-carbon units can enter the mitochondrial pteroylglutamate pool at the formyl and hydroxymethyl levels of oxidation. A number of experiments were therefore designed to examine ability of isolated pea mitochondria to generate, interconvert and transfer one-carbon units via the pteroylglutamate pool.

Biosynthesis of pteroylglutamates

In a preliminary experiment utilizing [*methylene*-^{14}C]-5,10-CH_2-H_4PteGlu as the substrate, it was found that the mitochondrial fraction could oxidize and reduce this derivative. Such interconversion suggests the presence of 5,10-CH_2-H_4PteGlu:NADP dehydrogenase and 5,10-CH_2-H_4PteGlu reductase, respectively. Substantial levels of label was also incorporated into other compounds not exchanging with DEAE-cellulose. Consequently further experiments were designed to investigate these labelling patterns more extensively using different C-1 donors.

When the mitochondrial fraction was incubated with [*methylene*-^{14}C]-5,10-CH_2-H_4PteGlu, radioactivity was incorporated into 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu (Table 8). In the presence of NADP or NAD the

TABLE 7. *Principal free amino acids of pea mitochondria*

Amino acid	nmoles
Lysine	5.56
Histidine	1.56
Aspartic	16.8
Threonine	16.1
Serine	97.5
Glutamic	112.0
Proline	20.5
Glycine	14.8
Alanine	28.9
1/2 Cysteine	1.61
Valine	28.6
Methionine	3.62
Isoleucine	5.23
Leucine	3.57
Tyrosine	0.63
Phenylalanine	0.04

The mitochondria were boiled in 80% (v/v) ethanol for 1 min and denatured protein was removed by centrifugation. The data are mean values of three separate analyses and are expressed as nmoles/g fresh weight of tissue.

TABLE 8. *Utilization of [methylene-^{14}C]-5,10-CH_2-H_4PteGlu by isolated mitochondria*

Reaction system	10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Serine	Methionine	Histidine	Unknown (1)
Complete	6970	1950	2280	1400	1970	2350
Substituted NAD for NADP	5050	950	4520	750	2280	2550
Substituted NADH for NADP	4940	1690	1730	420	2310	1010
Substituted NADPH for NADP	8090	2310	2620	550	3030	1110
Substituted NADH and $FADH_2$ for NADP	6200	17570	2620	770	18950	1225
Substituted NADH and homocysteine for NADP	5170	450	3320	2910	1210	3.5×10^6

The complete reaction system contained in 1 ml: 40 µmole KH_2PO_4 buffer (pH 7.5), 4 nmoles H_4PteGlu, 50 µCi [^{14}C]HCHO (15 µCi/µmole), 1 µmole NADP, 1 µmole 2-mercaptoethanol, 0.1 µmole EDTA and mitochondrial fraction. NAD, NADH, NADPH and FAD (1 µmole respectively) were substituted for NADP as additional controls. When NADH and FAD were combined, 0.3 units of Lipoyl dehydrogenase was added to produce an estimated initial concentration of 1 mM $FADH_2$. DL-homocysteine (1 µmole) was freshly prepared from the thiolactone. Data are expressed as cpm/g fresh weight of cotyledons.

labelled substrate was readily oxidized to 10-HCO-H_4PteGlu and smaller amounts of labelled 5-CH_3-H_4PteGlu were formed. Substitution of NADPH for NADP increased utilization of the substrate. The presence of $FADH_2$ did not decrease the amount of substrate oxidized to 10-HCO-H_4PteGlu but there was a marked increase in the labelling of the methyl derivative suggesting that 5,10-CH_2-H_4PteGlu reductase occurred in the mitochondrial fraction. When homocysteine was added, substantially less ^{14}C was recovered in 5-CH_3-H_4PteGlu. In addition to these pteroylglutamates, serine, methionine, histidine and an unidentified compound were synthesized (Table 8). Reducing conditions favored the production of histidine and substantial synthesis of the unidentified compound. The non-enzymic synthesis of this latter compound was ruled out as no label could be detected in identical control reaction systems where the mitochondrial fraction was omitted. When homocysteine was added, methionine synthesis was stimulated whereas the levels of ^{14}C incorporated into histidine were significantly decreased.

The ability of the isolated mitochondria to oxidize and reduce one-carbon units was further investigated by feeding [3-^{14}C]serine (Table 9). Results similar to the previous experiment were obtained with three exceptions. Firstly, when serine was the substrate the effects of adding $FADH_2$ and $FADH_2$ plus homocysteine, respectively, were different and NADPH was more effective in the synthesis of labelled 5-CH_3-H_4PteGlu. Secondly, the levels of ^{14}C in methionine were much lower than in the previous experiment and was possibly contingent upon the lack of effect of $FADH_2$. An interesting observation arising from this experiment was the synthesis of glycine. Thirdly, the levels of

TABLE 9. *Incorporation of [3-^{14}C]serine into derivatives of H_4PteGlu and free amino acids by isolated mitochondria*

Reaction system	10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Glycine	Methionine	Unknown (2)
Complete	13300	3000	*n.d.*	170	*n.d.*
Minus enzyme	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Substituted NADPH for NADP	14600	5700	1510	140	*n.d.*
Substituted NADPH and $FADH_2$ for NADP	14800	2400	1360	240	630
Substituted NADPH, $FADH_2$ and homocysteine for NADP	14200	2500	2090	170	*n.d.*

n.d. - not detectable.

The complete reaction system contained in 1 ml: 40 µmole KH_2PO_4 buffer (pH 7.5), 4 nmoles H_4PteGlu, 0.2 µmole pyridoxal-5'-phosphate, 1 µmole NADP, 4.8 µCi [3-^{14}C]serine (48 µCi/µmole), 1 µmole 2-mercaptoethanol, 0.1 µmole EDTA and mitochondrial extract. NADPH was substituted for NADP as a control. As an additional supplement to the reaction system, NADPH and FAD were combined with 0.3 units of Lipoyl dehydrogenase in the presence and absence of 1 µmole DL-homocysteine. Data are expressed as cpm/g fresh weight of cotyledons.

^{14}C incorporated into 10-HCO-H_4PteGlu were substantially higher than the levels of radioactivity in 5-CH_3-H_4PteGlu (Table 9) or the levels of radioactivity in 10-HCO-H_4PteGlu, when [*methylene*-^{14}C]-5,10-CH_2-H_4PteGlu was the immediate substrate (Table 8). This result is interesting as the incorporation of ^{14}C from [3-^{14}C]serine into derivatives of the pteroylglutamate pool must be limited initially by the activity of serine hydroxymethyltransferase. As [*methylene*-^{14}C]-5,10-CH_2-H_4PteGlu is a product of this reaction one might expect [^{14}C]HCHO to be a better precursor of these pteroylglutamates, especially when the specific activities of formaldehyde and serine are considered. These results might imply some structural organization which could effect carbon flow from C-1 donors, such as serine, glycine and formaldehyde, into the mitochondrial pteroylglutamate pool at the hydroxymethyl level of oxidation.

When the incorporation of [^{14}C]formate into H_4PteGlu derivatives and free amino acids was determined (Table 10), it was clear that in the presence of NADP all the radioactivity was apparently trapped in 10-HCO-H_4PteGlu. However, when conditions favored reduction, accumulation of 10-HCO-H_4PteGlu was not observed and some labelling of 5-CH_3-H_4PteGlu, methionine and serine occurred. In similar experiments employing [*methyl*-^{14}C]-5-CH_3-H_4PteGlu as substrate, incorporation of radioactivity into serine and methionine was also observed (Table 11).

Mitochondria incubated with [2-^{14}C]glycine (Table 12) incorporated label into 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu. In addition, some ability to methylate homocysteine was observed in this experiment. The labelling of pteroylglutamate derivatives in this experiment suggests that the mitochondria have some ability to cleave glycine

TABLE 10. *Incorporation of [^{14}C]formate into derivatives of H_4PteGlu and amino acids*

Reaction system	10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Serine	Methionine	Unknown (3)
Complete	*n.d.*	140	175	525	595
Minus enzyme	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
NADP substituted for NADPH	4940	*n.d.*	*n.d.*	*n.d.*	*n.d.*

n.d. - not detectable.

The complete reaction system contained in 1 ml: 22.7 µmole triethanolamine buffer (pH 8), 4 nmole H_4PteGlu, 4.38 µmole ATP, 1 µmole NADPH, 10 µmoles $MgCl_2$, 5.9 µCi [^{14}C]HCOOH (0.118 µCi/µmole) and mitochondrial extract. 1 µmole of NADP was substituted for NADPH as an additional supplement to the reaction system. HCOOH was titrated to pH 8 before addition to the reaction system. Data are expressed as cpm/g fresh weight of cotyledon.

TABLE II. *Incorporation of ^{14}C from [methyl-^{14}C]-5-CH_3-H_4PteGlu into free amino acids by the isolated mitochondria*

Reaction system	Methionine	Serine	Total ^{14}C in amino acids
Complete	280	235	515
Minus mitochondria	--	--	--
Plus diaphorase	560	105	665

The complete reaction system contained: 40 µmoles KH_2PO_4 buffer (pH 7.5), 2 µmoles H_4PteGlu, 3 µCi 5-$C^{14}H_3$-H PteGlu (61 µCi/µmole), 0.5 µmole FAD, 1 µmole NADP, 0.1 µmole EDTA, 2 µmoles menadione and mitochondrial fraction. Two units of lipoyl dehydrogenase was used as an additional supplement to the reaction system. Data are expressed as cpm/g fresh weight of cotyledons.

TABLE 12. *Incorporation of [2-^{14}C]glycine into derivatives of H_4PteGlu and amino acids*

Reaction system	10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	Serine	Methionine	Unknown (4)
Complete	760	280	101500	*n.d.*	3600
Minus enzyme	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Plus FAD and diaphorase	*n.d.*	100	4350	*n.d.*	360
Plus FAD, diaphorase and homocysteine	*n.d.*	370	7500	285	*n.d.*

n.d. - not detectable.

The complete reaction system contained in 1 ml: 40 µmole KH_2PO_4 buffer (pH 7.5), 4 nmole H_4PteGlu, 5.7 µCi [2-^{14}C]glycine (57 µCi/µmole), 1 µmole 2-mercaptoethanol, 2 µmoles dithiothreitol, 0.1 µmole pyridoxal-5'-phosphate, 1 µmole NAD and mitochondrial extract. FAD (0.5 µmole) combined with 0.3 units of lipoyl dehydrogenase in the presence and absence of 1 µmole DL-homocysteine were used as additional supplements to the reaction system. Data are expressed as cpm/g fresh weight of cotyledons.

molecules. This ability was examined in further experiments.

Partial characterization of glycine decarboxylase and the bicarbonate exchange reaction

In order to investigate the occurrence of a glycine cleavage reaction in mitochondria, an assay method was designed and applied to the mitochondrial fraction recovered by sucrose density gradient centrifugation. Sucrose (0.65 mM) was present in all reaction systems as an osmoticum because preliminary assays without sucrose yielded very low glycine decarboxylase or exchange activities.

The distribution of a glycine-bicarbonate exchange reaction (Scheme 2, reaction 1, p. 11) between soluble and particulate fractions is shown in Table 13. The mitochondrial fraction had approximately 47 times the specific activity of the unfractionated homogenate. Particulate enzyme activity was relatively stable during 24 h of storage at -10°C (Figure 5) but losses of activity were appreciable after longer periods of storage. Table 14 illustrates the requirements for bicarbonate, sulphydryl groups, pyridoxal phosphate and mitochondrial protein for optimal exchange of bicarbonate with the carboxyl carbon of glycine. Addition of H_4PteGlu reduced the amount of $^{14}CO_2$ recovered (Table 14) and disruption of mitochondrial integrity by various solubilization treatments also reduced exchange activity (Table 15).

The reaction proceeded under aerobic and anaerobic conditions (Table 16) but enzyme activity in air and O_2 was slightly higher than found in N_2. Under optimum assay conditions the rate of reaction was linear for at least 1 h (Figure 6A) and maximal exchange occurred at pH 7.8 (Figure 6B). The production of $^{14}CO_2$ from the exchange

TABLE 13. *The distribution of the [1-^{14}C]glycine-bicarbonate exchange reaction between soluble and particulate cell fractions*

	Total enzyme (nmole $^{14}CO_2$/ g fresh weight)	Specific activity (nmole $^{14}CO_2$/mg protein)	Enzyme recovery % of total
Crude homogenate	16.5	0.068	100
27,000*g* particulate fraction	0.82	0.727	5.0
Mitochondrial fraction	0.80	3.2	4.8

The complete reaction mixture, total volume 2 mls, containing: 1.3 µmole sucrose, 0.5 µCi [1-^{14}C]glycine (10 µmoles), 60 µmoles sodium bicarbonate, 100 µmoles Tris-HCl buffer (pH 7.8), 1.5 µmole pyridoxal phosphate, and 10 µmoles dithiothreitol, was incubated in air at 37°C for 30 min with 5 mg protein of the fractions indicated.

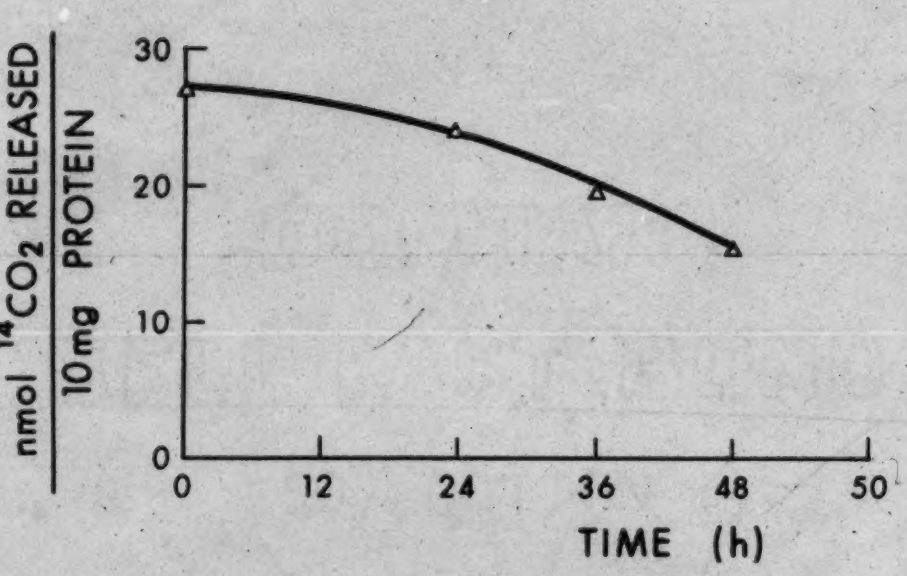


Figure 5. The effect of storage of the mitochondrial fraction on the activity of the [1-^{14}C]glycine-bicarbonate exchange reaction. Aliquots of the particulate fraction of the same protein concentration were stored at -10°C and thawed for assay when needed. The complete assay system was the same as that described in Table 13.

TABLE 14. *Requirements of the glycine-bicarbonate exchange reaction*

Omission from the reaction system	nmole of $^{14}CO_2$ recovered
None	28.3
Mitochondrial fraction	--
DTT	22.1
Pyridoxal phosphate	12.3
Bicarbonate	5.6
None plus FAD (1 µmole)	16.1
None plus H_4PteGlu (0.2 µmole)	21.7

The complete reaction conditions are described in Table 13 with the exception that the flasks were flushed with N_2 before incubation. The data are expressed as nmole $^{14}CO_2$ recovered per 10 mg protein per 30 min.

TABLE 15. *The effect of detergents and mechanical treatment of the mitochondrial fraction on the glycine-bicarbonate exchange reaction*

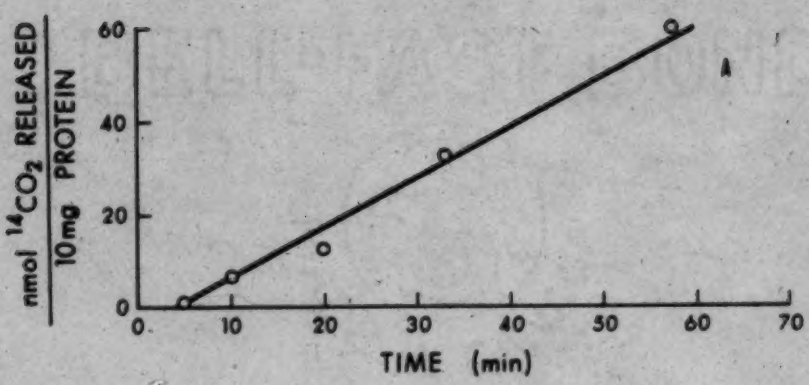
Treatment	nmole of $^{14}CO_2$ recovered
None	32.0
Triton X 100 (0.1%)	10.2
Digitonin (0.1%)	11.1
Deoxycholate (0.1%)	7.5
Sonication	10.1

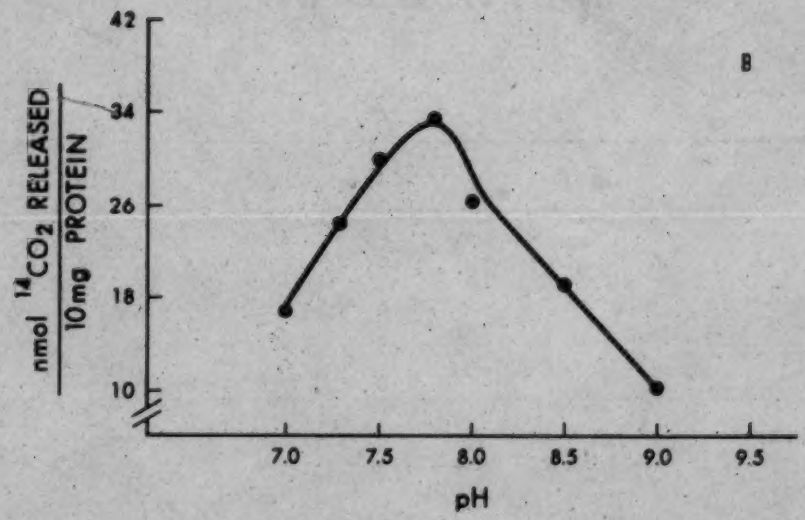
The solubilization treatments were carried out for 2 min at 0°C using the following solubilizer or detergent: Triton X 100 (0.1% v/v), digitonin (0.1% w/v) or deoxycholate (0.1% w/v). The effect of mechanical disruption was examined by sonicating the isolated mitochondria at full amplification for 1 min at 4°C with a Fisher Ultrasonic Generator. The complete reaction conditions are described in Table 13. The data are expressed as nmole $^{14}CO_2$ recovered per 10 mg protein per 30 min.

TABLE 16. *Effects of oxygen tension on the exchange reaction catalyzed by the isolated mitochondria*

Gas phase	nmole of $^{14}CO_2$ recovered
N_2	22.7
Air	26.4
O_2	26.2

After the cofactors, enzyme and [1-^{14}C]glycine were added to the reaction flask as described in Table 13, the atmosphere of each flask was quickly flushed as indicated. The data are expressed as nmole $^{14}CO_2$ recovered per 10 mg protein per 30 min.





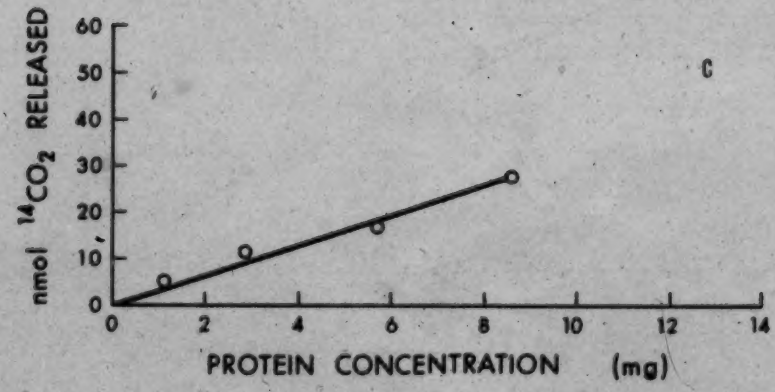


FIGURE 6. Production of $^{14}CO_2$ from the exchange reaction as a function of: time, pH or protein concentration.

The reaction conditions are similar to those described in Table 13 with the following exceptions: the time of incubation at 37°C (Figure A), the pH of Tris HCl buffer (Figure B) or, the amount of mitochondrial protein added (Figure C), was varied as illustrated.

reaction was linearly increased by varying the concentration of mitochondrial protein in the reaction system (Figure 6C). Product formation also increased linearly with bicarbonate concentrations up to 18 mM and with glycine concentrations up to 1.1 mM. Lineweaver-Burk (1934) plots for these substrates gave apparent K_m values of 12.5 mM and 1.8 mM respectively (Figures 7A and B). These values are similar in magnitude to those reported by Sato *et al.* (1969) for enzyme preparations of rat liver.

The glycine cleavage or decarboxylase reaction, assayed in the absence of bicarbonate, could be stimulated by NAD^+. Product formation increased linearly with NAD^+ concentrations up to 30 µM. A Lineweaver-Burk (1934) plot for this substrate gave an apparent K_m value of 47 µM (Figure 7C). The cleavage of glycine was suppressed by reduced pyridine nucleotides (Figure 8).

The effects of solubilization treatments on enzyme activity together with the apparent aerobic stimulation and inhibition by reduced pyridine nucleotides, implies that a suitable oxidation-reduction balance, possibly mediated by the mitochondrial membranes, is of importance in this reaction. In this regard, Bird *et al.* (1972) have reported that the conversion of two glycine molecules to serine by crude preparations of tobacco leaf mitochondria, requires O_2, is inhibited by inhibitors of mitochondrial electron transport and is stimulated by ADP. These authors have concluded that ATP was synthesized at the expense of free energy derived from the cleavage of glycine. Attempts to show such an effect in pea mitochondria were not successful (Figure 9). It is possible that further control of this

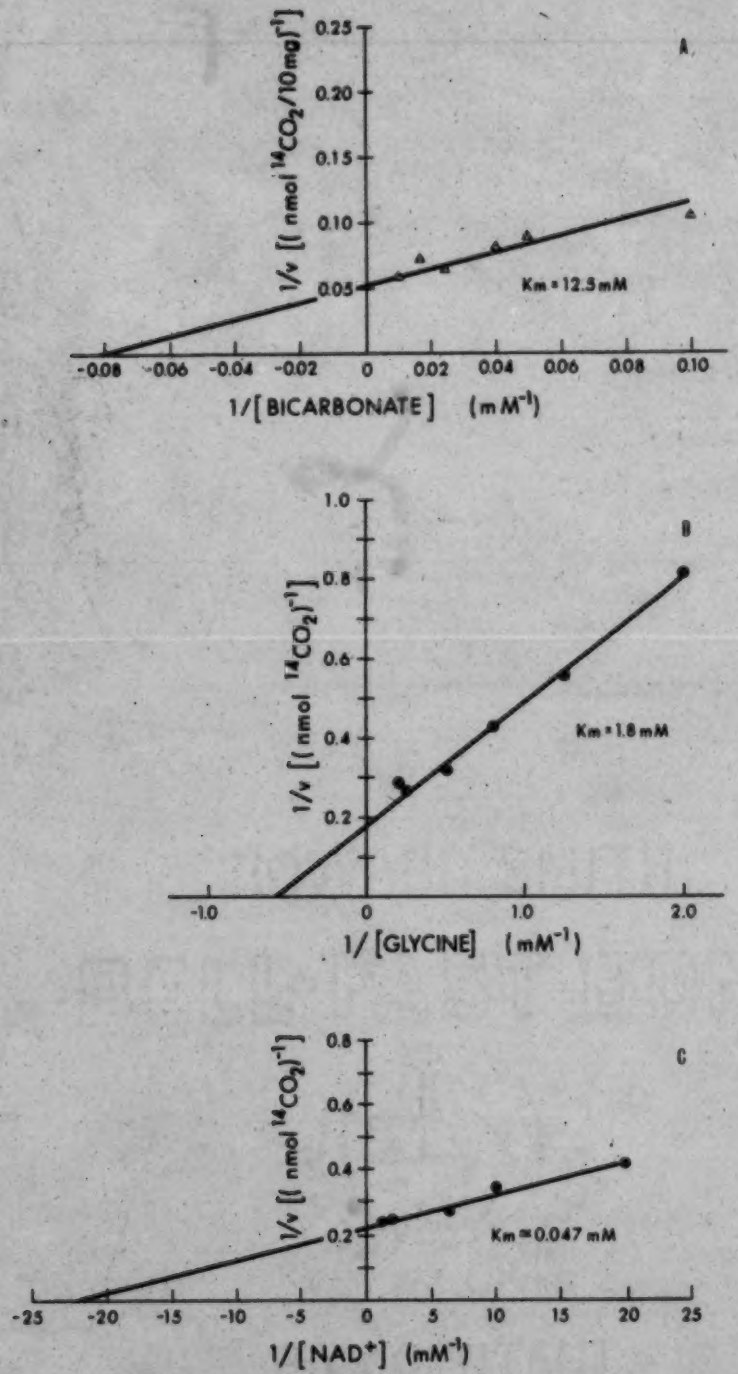


FIGURE 7. Double reciprocal plots of $^{14}CO_2$ produced *versus* the concentration of: bicarbonate, glycine or NAD^+.

The complete reaction mixture is described in Table 13 with the following exceptions: the concentration of bicarbonate (Figure A), the concentration of glycine (Figure B) or, the concentration of NAD^+ (Figure C), was varied as illustrated. Furthermore, when NAD^+ was added for assay of the glycine cleavage reaction, bicarbonate was omitted as described in the text.

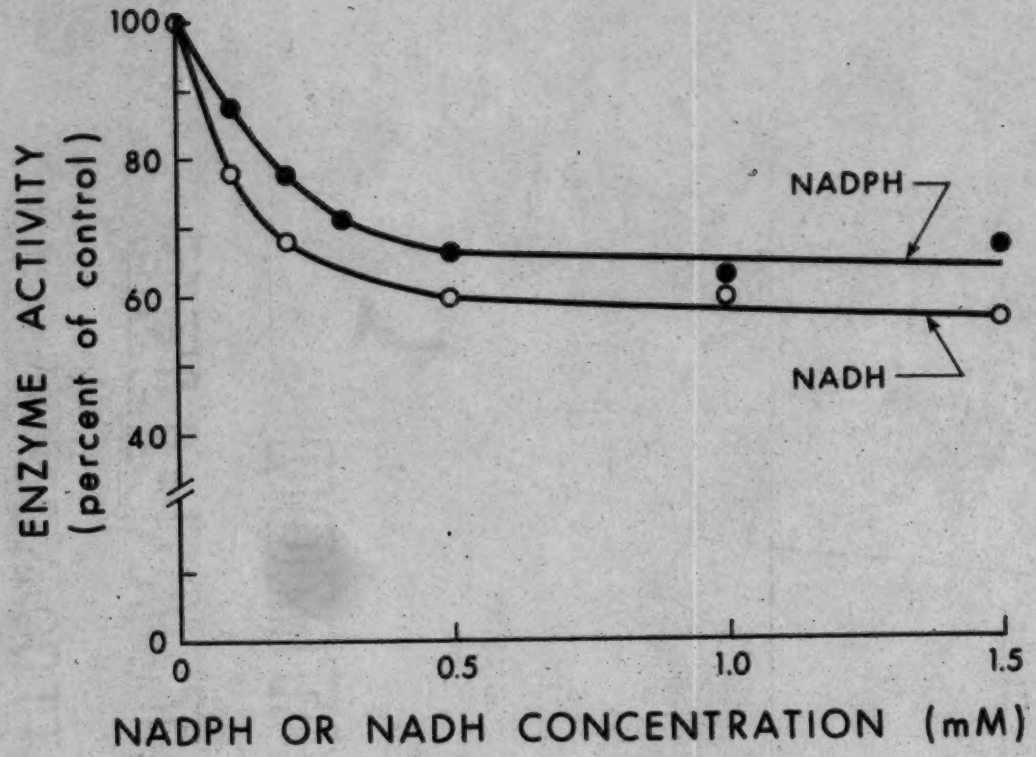


FIGURE 8. Reduced pyridine nucleotide inhibition of glycine decarboxylase activity. The complete reaction system is described in the methods section. NADH or NADPH was added in the concentrations illustrated. The control reaction system produced 3.5 nmoles of $^{14}CO_2$ per mg of protein.

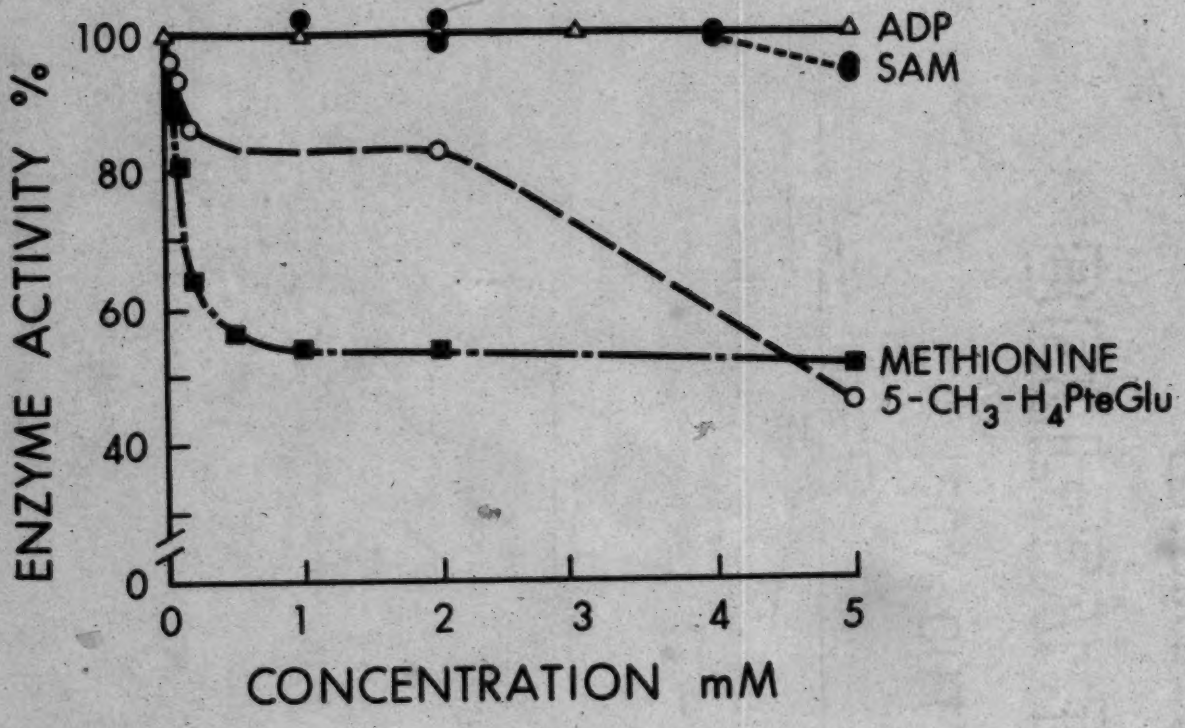


FIGURE 9. The effect of L-methionine, 5-CH_3-H_4PteGlu, SAM and ADP on glycine decarboxylase activity.
Supplements were added to give the concentrations illustrated in the complete reaction system (see Table 13).

reaction may be affected by various intermediates and products of C-1 metabolism. In this respect, methionine and 5-CH_3-H_4PteGlu were both effective in reducing the activity of glycine decarboxylase *in vitro* whereas, S-adenosylmethionine was not (Figure 9).

In animal tissues glycine cleavage is a significant catabolic process resulting in complete oxidation of the molecule to CO_2. Because such oxidation necessitates involvement of the pteroyl-glutamate pool, an experiment was designed to determine if the mitochondria had ability to convert [2-^{14}C]glycine to $^{14}CO_2$. Table 17 illustrates that when [2-^{14}C]glycine with a high specific activity was incubated with the isolated mitochondria, label from the second carbon was incorporated into CO_2. These results, coupled with the labelling of 10-HCO-H_4PteGlu (Table 12) might imply the activity of 10-HCO-H_4PteGlu:NADP oxidoreductase in the isolated mitochondria.

Homocysteine-dependent transmethylase activity

From the data presented (Tables 8 to 12), it follows that mitochondria isolated from germinating pea cotyledons possess ability to transmethylate homocysteine. The occurrence of such a mitochondrial transmethylase together with the mitochondrial system for generation of methyl groups clearly points to a compartmentation of methionine biosynthesis in pea cotyledons. Previous studies from this laboratory (Dodd and Cossins, 1970) have examined homocysteine-dependent transmethylase activities of pea cotyledon homogenates. Consequently it was of interest to examine mitochondria for these enzymes in the present work.

TABLE 17. *The conversion of [2-^{14}C]glycine to $^{14}CO_2$ by isolated pea mitochondria*

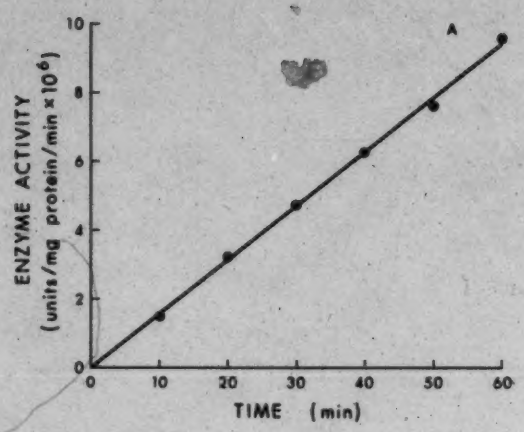
Reaction system	nmole of $^{14}CO_2$
Complete	0.016
Complete + NADP	0.014
Complete + NADP + H_4PteGlu	0.014
Control	nil

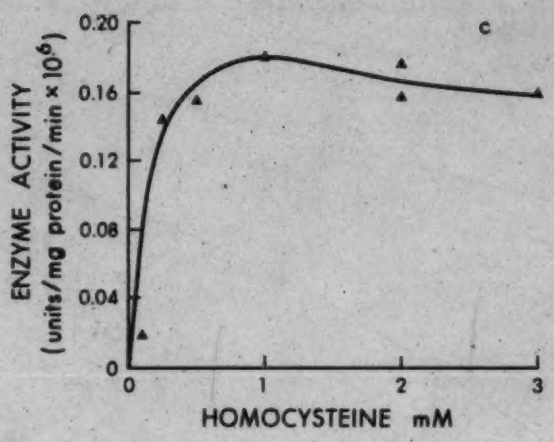
The mitochondrial fraction was incubated for 30 min at 37°C under the conditions employed in the glycine decarboxylase assay. The complete reaction system in a total volume of 2 mls contained: 4 µCi glycine (21.8 µCi/µmole), 1 mmole sucrose, 1.5 µmole pyridoxal phosphate, 100 µmoles Tris-HCl buffer (pH 7.8), 1.25 µmole NAD, 10 µmoles DTT and mitochondrial fraction (10 mg protein). NADP (6.2 µmoles) and H_4PteGlu (1 µmole) were added as supplements to the reaction system. Control reaction systems did not contain the mitochondrial fraction.

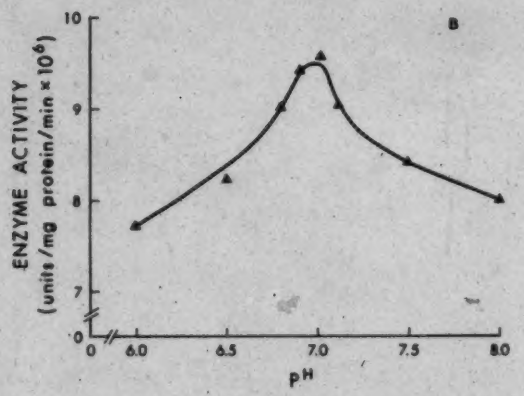
Two distinct homocysteine-dependent transmethylases were present in mitochondrial protein precipitating between 20 and 60% of saturation with ammonium sulphate (Table 18). Total activity of the transmethylase utilizing 5-CH_3-H_4PteGlu as the methyl donor was substantially greater than the activity of the SAM transmethylase in both the cotyledon homogenate and the particulate fractions. Some 44.7% of the total activity of the former enzyme was present in the ammonium sulphate fraction of the isolated mitochondria (Table 18). The 5-CH_3-H_4PteGlu enzyme was also purified approximately ten fold, during its isolation. A substantially lower yield and purification of the SAM transmethylase was noted (Table 18).

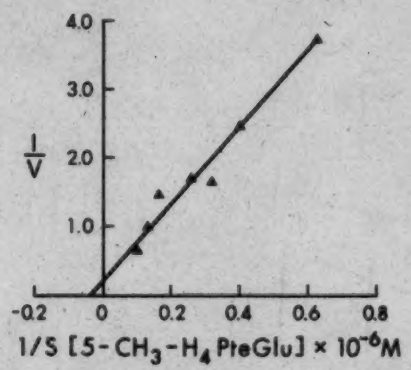
Product formation by both enzymes was linear for over 1 h (Figure 10A), consequently, a 1 h incubation period at 30°C was employed in further experiments. The pH optima of 7 for both particulate enzymes (Figure 10B) enabled simple comparisons between their apparent catalytic properties, at pH 6.9, and those reported for the enzymes isolated from cotyledon homogenates by Dodd and Cossins (1970) (Table 19).

The 5-CH_3-H_4PteGlu transmethylase prepared from the isolated mitochondria was saturated with homocysteine at a concentration of approximately 1.0 mM (Figure 10C). The corresponding transmethylase from cotyledon homogenates was saturated with homocysteine at a concentration of 2 mM. The apparent K_m value (25 µM) for the methyl donor, determined from a Lineweaver-Burk plot (Figure 10D), revealed that the 5-CH_3-H_4PteGlu transmethylases from both cell fractions had similar affinities for the methyl donor (Table 19). The apparent Michaelis constant for the mitochondrial SAM transmethylase









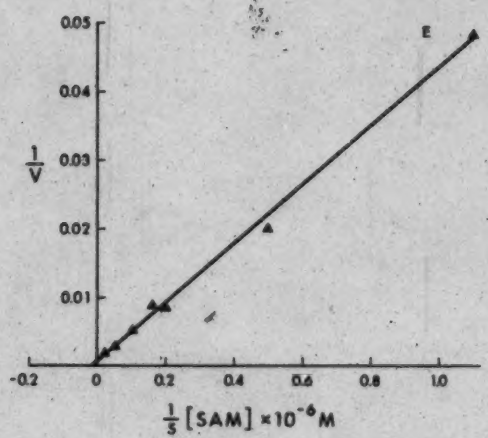


FIGURE 10. Production of [methyl-^{14}C]-L-methionine by mitochondrial transmethylases. Product formation by the 5-CH_3-H_4PteGlu transmethylase was linear with time as illustrated (Figure A). The pH optima of this reaction was determined by varying the pH of the buffer as illustrated (Figure B). Saturating concentrations of homocysteine were obtained by varying the concentration of homocysteine in the standard reaction mixture as illustrated (Figure C). Lineweaver-Burk plots revealed apparent Michaelis constants of 25 μM for 5-CH_3-H_4PteGlu (Figure D) and 50 μM for SAM (Figure E) for the 5-CH_3-H_4PteGlu and SAM transmethylases, respectively. All assays were carried out at 30°C as previously described utilizing a 20-60% $(NH_4)_2SO_4$ fraction of the isolated mitochondria as the source of enzyme.

TABLE 18. *Fractionation of extracts catalyzing homocysteine-dependent transmethylation reactions utilizing [methyl-^{14}C]-5-CH_3-H_4PteGlu or SAM-[methyl-^{14}C] as methyl donors*

Cell fraction	Total enzyme (units x 10^6)	Specific activity (units/mg protein x 10^6)	Purification factor	Enzyme recovery (% of total units)
Whole cotyledon homogenate				
Methyl donor:				
5-CH_3-H_4PteGlu	21.5	8.4	1	100
SAM	9.8	0.45	1	100
Mitochondrial fraction 20-60% $(NH_4)_2SO_4$ preparation				
Methyl donor:				
5-CH_3-H_4PteGlu	11.6	87	10.3	44.7
SAM	0.13	0.47	1.05	1.3

Homocysteine-dependent methyltransferase activities were measured in a reaction system of 0.5 ml containing: 1 mg enzyme preparation, 1 µmole L-homocysteine, 1.6 nmole [*methyl*-^{14}C] (1.82 x 10^5 dpm) and 50 µmoles potassium phosphate buffer (pH 6.9). The data given are calculated on a per g fresh weight of tissue basis. One unit of enzyme activity is the amount of enzyme catalyzing the production of 1 µmole of L-methionine in 1 min at 30°C.

TABLE 19. *Comparison of homocysteine-dependent transmethylases from whole cotyledon homogenates*[a] *and mitochondrial preparations*

Preparations	Saturating concentration with homocysteine (mM)	Apparent Michaelis constant for the methyl donor (μM)	Concentration of methionine producing 50% inhibition (mM)
Cotyledon homogenate after Sephadex G-100 fractionation			
Methyl donor:			
5-CH_3-H_4PteGlu	2.0	26	3.0
SAM	2.0	4	>10.0
Mitochondrial fraction after 20-60% $(NH_4)_2SO_4$ fractionation			
Methyl donor:			
5-CH_3-H_4PteGlu	1.0	25	2.5
SAM	--	50	--

[a] All data for cotyledon extracts are from Dodd, W.A. (1969) Ph.D. Thesis, Department of Botany, University of Alberta, published in part in *Biochim. Biophys. Acta* 201, 261 (1970).

was 50 μM (Figure 10E).

Product inhibition of homocysteine-dependent transmethylases by L-methionine is now well known (Dodd and Cossins, 1970; Abramson and Shapiro, 1965; Cossins *et al.*, 1972) and was displayed by the 5-CH_3-H_4PteGlu transmethylase of pea mitochondria (Figure 11). Relatively low concentrations of L-methionine inhibited 5-CH_3-H_4PteGlu transmethylase activity, the concentrations of this amino acid giving 50% inhibition of the enzymes from both cell fractions being similar (Table 19). Due to this effect of methionine it is possible that estimates of total 5-CH_3-H_4PteGlu transmethylase activity in the initial unfractionated cotyledon homogenates are low.

Biosynthesis of S-adenosylmethionine by pea mitochondria

The principal mechanisms for biosynthesis of SAM involve the methylation of S-adenosylhomocysteine and the activation of methionine by ATP. Dodd and Cossins (1969) have concluded that SAM is formed in pea cotyledons by a methionine activating enzyme utilizing ATP. A similar synthesis of SAM by preparations of isolated pea mitochondria was observed in the present work (Table 20). Maximal synthesis of SAM by these preparations was dependent on ATP and Mg^{++}, as well as the presence of a thiol group (Table 20). In contrast, the possible synthesis of S-methylmethionine by the mitochondrial preparations (reaction system described on page 34) could not be shown, implying that SMM may not be generated in pea mitochondria.

A comparison of the levels of SAM previously reported for this tissue (0.04 μmoles per gram fresh weight; Dodd and Cossins, 1968) with the quantities synthesized in the present study (approximately 0.244

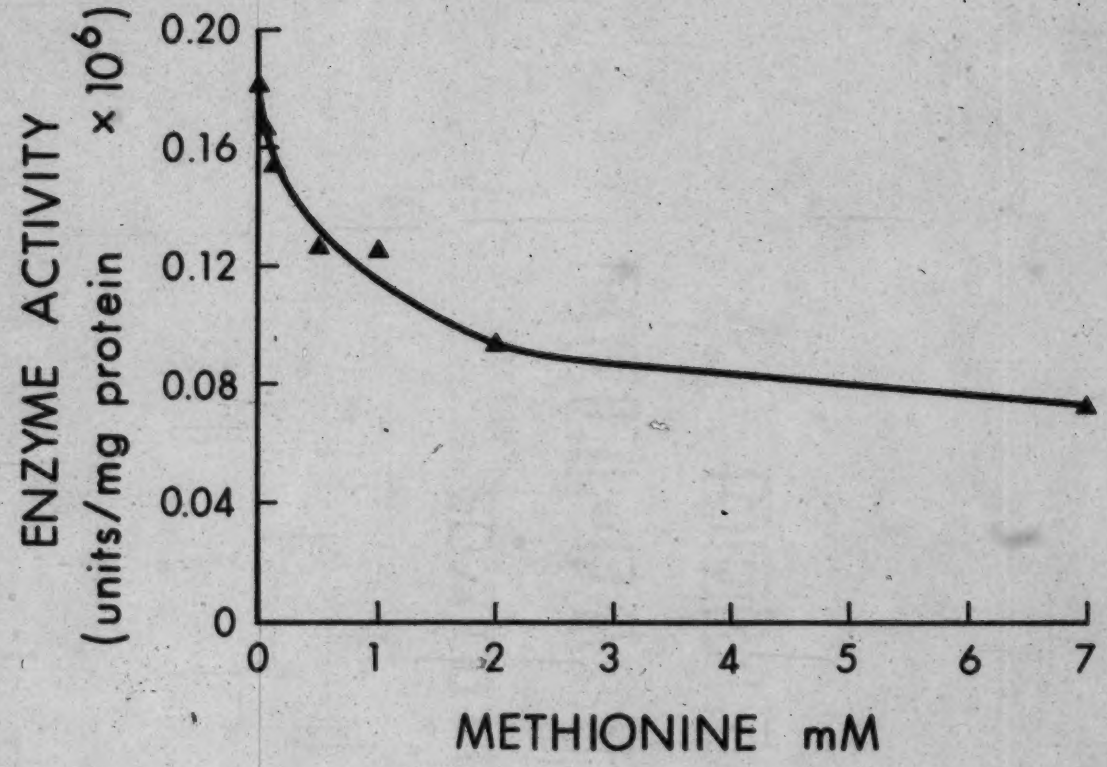


FIGURE 11. Product inhibition of the 5-CH_3-H_4PteGlu:homocysteine transmethylase.
Varying concentrations of L-methionine were added to the standard reaction mixtures as illustrated.

TABLE 20. *Synthesis of SAM by isolated mitochondria*

Omission from reaction system	SAM formed (µmole)
None	1.920
ATP	0.432
Mg^{++}	0.425
2-Mercaptoethanol	0.760
Mitochondrial fraction	0.011

The complete assay system contained in a total volume of 2 mls: 8 µmoles [*methyl*-^{14}C]-L-methionine (0.125 µCi/µmole), 10 µmoles ATP, 5 µmoles 2-mercaptoethanol, 100 µmoles potassium phosphate buffer (pH 6.9), 50 µmoles $MgCl_2$ and 2 mg mitochondrial protein (20-60% $(NH_4)_2SO_4$ fraction). The reaction mixtures were incubated for 60 min at 30°C.

μmoles per gram fresh weight) indicates that the mitochondria may be capable of synthesizing a large part of the SAM required in the metabolic activities of this tissue.

Homocysteine biosynthesis by isolated pea mitochondria

The observation that pea mitochondria have ability to metabolize and synthesize several amino acids indicates that this organelle has a significant role in the biogenesis of methyl groups for the *de novo* synthesis of methionine and SAM during germination. Regulation of mitochondrial synthesis of methionine and SAM in this compartmented system could conceivably be achieved at the sites of transmethylation and generation of methyl groups. As the transmethylation reaction represents a convergence in two synthetic pathways consideration of a third factor, namely the biosynthesis of homocysteine and hence transsulphuration must also be accounted for. Dodd and Cossins (1969) have presented a scheme of reactions to account for the possible recycling of sulphur in this tissue during germination. To the author's knowledge, no studies to date have examined the possibility of a compartmented system for homocysteine biosynthesis in plants.

Assays of β-cystathionase (Scheme 4, reaction 12, p.80), cystathionine-γ-synthase (Scheme 4, reaction 13) and L-homoserine transacetylase (transsuccinylase) (Scheme 4, reaction 14) were performed on protein fractions of isolated mitochondria as shown in Table 21. The cellular distribution of these enzymes in the mitochondria of 1.34%, 1.25%, 2.7% and 1.1% of total activity respectively, implies that the mitochondria may not be the only site for these reactions. However, the presence of these enzymes in isolated mitochondria indicates an

TABLE 21. *Localization of β-cystathionase, cystathionine-γ-synthase, L-homoserine transacetylase and L-homoserine transsuccinylase*

Cell fraction	Specific activity nmole product/mg protein	Total activity nmole/g fresh weight	Distribution %
Crude homogenate			
β-cystathionase	2.62	127	100
cystathionine-γ-synthase	0.403	19.2	100
L-homoserine transacetylase	0.064	1.82	100
L-homoserine transsuccinylase	0.062	1.76	100
Mitochondrial fraction			
β-cystathionase	3.20	1.7	1.34
cystathionine-γ-synthase	0.435	0.24	1.25
L-homoserine transacetylase	0.079	0.051	2.7
L-homoserine transsuccinylase	0.027	0.018	1.1
0-45% $(NH_4)_2SO_4$ fraction of isolated mitochondria			
β-cystathionase	3.37	0.873	0.69
cystathionine-γ-synthase	0.580	0.14	0.73

Enzyme activities are expressed as the amount of pyruvate formed by β-cystathionase and the amount of α-ketobutyrate formed by cystathionine-γ-synthase, per 10 min at 37°C under the reaction conditions described in the Materials and Methods. Activities of homoserine transacetylase and homoserine transsuccinylase are given as the amounts of N-acetylhomoserine and N-succinylhomoserine recovered respectively after 0.5 h incubation at 37°C.

ability to generate nanomolar quantities of homocysteine from homoserine. It is of interest to note that S-acetyl CoA was utilized more readily in the acylation of L-homoserine than was S-succinyl CoA (Table 22), suggesting that O-acetyl-L-homoserine may be the substrate for the cystathionine-γ-synthase reaction of mitochondria. End product inhibition of L-homoserine transacetylase, cystathionine-γ-synthase and β-cystathionase by L-methionine up to concentrations of 1 mM could not be demonstrated for the enzyme preparations studied (Table 23). The apparent lack of product inhibition by L-methionine does not rule out other regulatory mechanisms or perhaps feed-back inhibition mediated by other compounds such as SAM as observed for *Neurospora* (Kerr and Flavin, 1969; Shelhub *et al.*, 1971). If extensive recycling of sulphur occurs within the mitochondrion it is clear that the possible role of S-adenosylhomocysteine as a feed-back inhibitor would be worthy of investigation.

The presence of these enzymes also implies that the trans-sulphuration pathway (Scheme 4, reactions 12, 13 and 14) rather than the direct sulphydration of L-homoserine or its derivative, is the more likely pathway of homocysteine biosynthesis in pea mitochondria. The levels of L-homoserine transacetylase, cystathionine-γ-synthase and β-cystathionase also argue for a sufficient endogenous synthesis of homocysteine to accomodate the mitochondrial homocysteine-dependent transmethylation reactions.

TABLE 22. *Synthesis of O-acetyl-L-homoserine and O-succinyl-L-homoserine by isolated mitochondria*

Omission from reaction system	O-acetylhomoserine produced (nmole)
Complete	0.55
Mitochondrial fraction	nil
S-acetyl-CoA	0.001
S-acetyl CoA with S-succinyl CoA added	0.19

The complete assay system (total volume 0.5 ml) contained: 50 µmoles potassium phosphate buffer (pH 7.4), 0.5 µmole S-acetyl-CoA, 50 nmoles [U-^{14}C]-L-homoserine (1 µCi/µmole) and 7 mg mitochondrial protein. Reaction mixtures were incubated at 37°C for 0.5 h.

TABLE 23. *Lack of inhibition of L-homoserine transacetylase, cystathionine-γ-synthase and β-cystathionase by L-methionine*

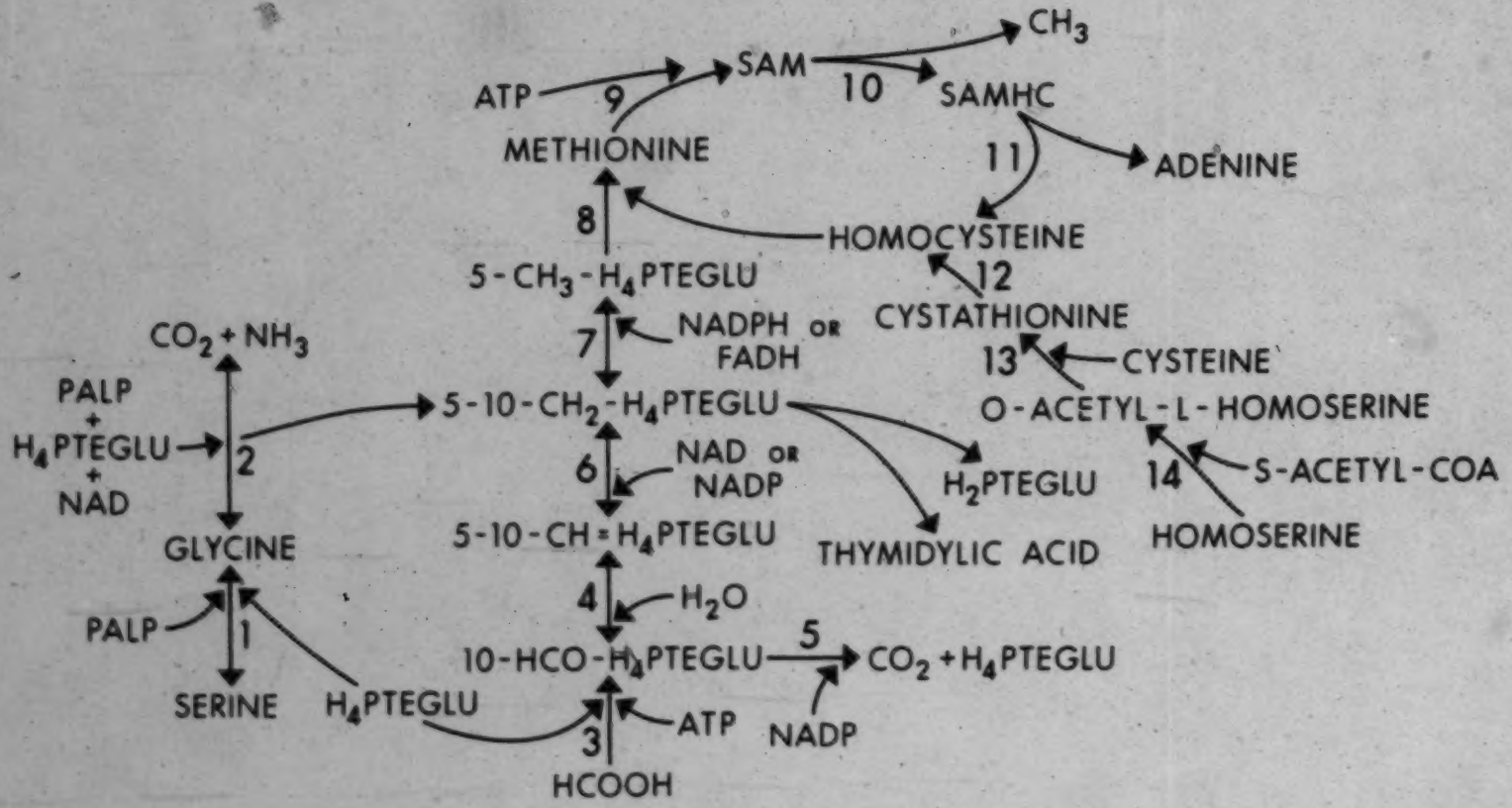
	Enzyme activity nmole of product/mg protein			
Concentration of L-methionine (mM)	0	0.01	0.1	1.0
L-homoserine transacetylase	0.079	0.080	0.079	0.081
Cystathionine-γ-synthase	0.580	0.581	0.584	0.579
β-cystathionase	3.37	3.38	3.36	3.34

Enzyme activities are expressed as the amount of pyruvate formed by β-cystathionase and the amount of α-ketobutyrate formed by cystathionine-γ-synthase, per 10 min at 37°C under the reaction conditions described in the Materials and Methods. Activities of homoserine transacetylase are given as the amount of N-acetylhomoserine recovered after 0.5 h incubation at 37°C.

SCHEME 4

Major C-1 transfer reactions leading to serine and methionine biosynthesis in plant mitochondria

Systematic enzyme name	Trivial name	EC number	Reaction
L-serine:H_4PteGlu-5,10-hydroxymethyltransferase	Serine hydroxymethyltransferase	2.1.2.1	1
Glycine:carboxylase	Glycine decarboxylase	4.1.99	2
Formate:H_4PteGlu ligase (ADP)	10-Formyltetrahydrofolate synthetase	6.3.4.3	3
5,10-CH=H_4PteGlu-5-hydrolase (decyclizing)	Cyclohydrolase	3.5.4.9	4
10-HCO-H_4PteGlu:NADP oxidoreductase	10-Formyltetrahydrofolate oxidoreductase	1.5.1.99	5
5,10-CH_2-H_4PteGlu:NADP oxidoreductase	5,10-Methylenetetrahydrofolate dehydrogenase	1.5.1.5	6
5-CH_3-H_4PteGlu:NADP (FAD) oxidoreductase	5,10-Methylenetetrahydrofolate reductase	1.1.1.68	7
5-CH_3-H_4PteGlu:homocysteine methyltransferase	5-methyltetrahydrofolate:homocysteine transmethylase	2.1.99	8
ATP:methionine S-adenosyltransferase	S-adenosyl-methionine synthetase	2.5.1.6	9
S-adenosyl-L-methionine: methyltransferase	S-adenosyl-methionine transmethylase	2.1.1.99	10
Cystathionine hydrolase	β-cystathionase	3.1.2.99	12
O-acetyl-L-homoserine hydrolyase	L-cystathionine-γ-synthase	4.2.1.13	13
Acetyl-CoA:L-homoserine S-acetyltransferase	L-homoserine transacetylase	2.3.1.99	14


CH3
SAM
ATP
9
10
SAMHC
METHIONINE
11
ADENINE
8
HOMOCYSTEINE
5-CH3-H4PTEGLU
12
CYSTATHIONINE
7
NADPH OR FADH
13
CYSTEINE
CO2 + NH3
PALP + H4PTEGLU + NAD
2
5-10-CH2-H4PTEGLU
O-ACETYL-L-HOMOSERINE
6
NAD OR NADP
H2PTEGLU
14
S-ACETYL-COA
GLYCINE
5-10-CH=H4PTEGLU
THYMIDYLIC ACID
HOMOSERINE
4
H2O
PALP
1
10-HCO-H4PTEGLU
5
CO2 + H4PTEGLU
SERINE
H4PTEGLU
3
ATP
NADP
HCOOH

SCHEME 4

DISCUSSION

Evidence for metabolic compartments which are related to the turnover of pools of many metabolites in the cell is now available for numerous plant species (see reviews by Steward and Bidwell, 1962; Laties, 1969; Britten and McClure, 1962; Beevers *et al.*, 1966; Mandelstom, 1960; Oaks and Bidwell, 1970). Oaks and Bidwell (1970) contend that such compartments isolate metabolites participating in competing metabolic sequences and regulate their critical levels thus permitting selective transfer of these metabolites for the ordered progress of cell metabolism. Such a compartmented system for the metabolism of pteroylglutamate derivatives and some associated reactions is indicated by the present study.

Compartmentation of pteroylglutamate derivatives

On the basis of widely accepted criteria, it is reasonable to conclude that fraction 5 is essentially mitochondrial (Table 3). The presence of enzymes such as serine hydroxymethyltransferase (Scheme 4, reaction 1) and 10-HCO-H_4PteGlu synthetase (Scheme 4, reaction 3) in the isolated mitochondria, suggests that at least part of the one-carbon metabolism of this tissue is compartmented. This possibility is substantiated by the presence, in this fraction, of pteroylglutamate derivatives, known to be metabolically important in other tissues (Tables 4 and 5). This contention is also supported by the solubilization studies (Table 6) which indicated that the derivatives associated with the mitochondria may be differentially bound. Conceivably this

binding may result in a spatial organization of the mitochondrial pteroylglutamate pool, which could have considerable physiological significance. In this connection, it is of interest to note that mitochondria from other species contain a number of metabolically important pteroylglutamates and in some cases ability to interconvert these has been clearly demonstrated (Noronha and Sreenivasan, 1960; Wang *et al.*, 1967; Sankar *et al.*, 1969). It is, therefore, logical to conclude that the association and possibly metabolism of certain pteroylglutamates in mitochondria is ubiquitous to higher organisms.

Examination of the degree of conjugation of the pteroylglutamate derivatives present in the isolated mitochondria was complicated by mitochondrial hydrolase activity. Incubation of yeast extract with isolated mitochondria resulted in a ten-fold increase in the growth response of *P. cerevisiae* implying that mitochondria were active in hydrolyzing the highly conjugated pteroylglutamate derivatives present. It is, therefore, apparent that the degree of conjugation of the pteroylglutamate derivatives present in isolated mitochondria (Table 4 and Figure 3) may not be completely representative of the polyglutamyl forms that may be present in this organelle *in vivo* or before isolation. In this respect it was apparent from column chromatography of the mitochondrial fraction (Figure 3) that polyglutamyl derivatives greater than the di- or triglutamyl level of conjugation were a minor component of the mitochondrial pteroylglutamate pool after isolation of this organelle.

When expressed on a fresh weight basis the levels of mitochondrial formyl derivatives were found to be approximately 18.0 ng/g fresh weight compared with the levels of 105 ng/g fresh weight for the whole tissue

extracts. Clearly approximately 17% of the formyl pool was associated with the isolated mitochondria. This value is, however, a minimal one as formyl derivatives would undoubtedly be lost to the supernatant during isolation of the organelle (Tables 4 and 6). Similar calculations of the distribution of methyl derivatives revealed that they may be largely associated with the soluble components of the cell or very rapidly utilized in the mitochondria.

Recent studies by Roos and Cossins (1971) have demonstrated the labelling of 5-CH_3-H_4PteGlu from [2-^{14}C]PteGlu in the cotyledon of germinating pea seeds, though no ^{14}C could be detected in the substantial pools of 10-HCO-H_4PteGlu isolated. These and the observations previously discussed are quite consistent with, and even clarified by, the apparent compartmentation of formyl derivatives demonstrated for cotyledon mitochondria (Clandinin and Cossins, 1972).

Associated reactions of C-1 metabolism in isolated mitochondria

Upon consideration of the numerous reactions in which pteroylglutamate derivatives participate, it is apparent that information regarding the intracellular localization, rates of turnover and metabolism of these derivatives will have considerable impact upon the understanding of the catabolic and biosynthetic processes that are known to occur in the germinating seed and in particular, the mitochondria.

It is clear from Tables 8 and 9 that isolated pea mitochondria are capable of synthesizing formyl and methyl pteroylglutamates from 5,10-CH_2-H_4PteGlu (Scheme 4, reactions 6 and 7, respectively). Such syntheses would involve oxidation and reduction of one-carbon units.

In addition, synthesis of associated amino acids, as summarized in Scheme 4 was observed (Tables 8, 9 and 10). When one-carbon units were supplied at the hydroxymethyl level of oxidation it was apparent that subsequent oxidation of the C-1 unit was favored when NAD or NADP was present (Tables 8 and 9). Substitution of NADH or NADPH for NADP resulted in increased utilization of the substrate supplied and favored reduction of the one-carbon unit to the methyl level of oxidation. The presence of reduced FAD in these reaction systems increased the incorporation of label into amino acids, particularly methionine and an unidentified compound when homocysteine was also supplied (Tables 8 and 9).

The homocysteine-dependent synthesis of methionine and production of serine and histidine (Tables 8 to 12) further implicate the mitochondria in the biosynthesis of amino acids related to pteroylglutamate metabolism. Furthermore, the substantial incorporation of ^{14}C into a number of unidentified compounds, particularly under conditions which favor methionine synthesis (Table 8), suggests the operation of other pathways related to mitochondrial pteroylglutamate metabolism. These other products were not formed in reaction systems containing boiled mitochondrial fraction. However, considering the very high levels of ^{14}C incorporated in some cases (Table 8), the possibility of non-enzymic reactions cannot be entirely ruled out.

Isolated pea mitochondria also synthesized 5-CH_3-H_4PteGlu from 10-HCO-H_4PteGlu (Table 10), an ability apparently lacking in rat liver mitochondria (Wang *et al.*, 1967). In the presence of NADP, when the reaction conditions favored oxidation, no synthesis of 5-CH_3-H_4PteGlu occurred indicating that 5,10-CH_2-H_4PteGlu dehydrogenase and reductase

were both instrumental in catalyzing this conversion.

The apparent absence of 5,10-CH_2-H_4PteGlu dehydrogenase and 5,10-CH_2-H_4PteGlu reductase in rat liver mitochondria led Wang *et al.* (1967) to conclude that the major role of mitochondrial serine hydroxymethyltransferase was related to the reversible interconversion of glycine and serine. However, it seems likely in pea mitochondria that serine hydroxymethyltransferase, besides functioning in the interconversion of serine and glycine, may act as a source of C-1 units for utilization by the mitochondrial pteroylglutamate pool. Clearly the extensive metabolism of the one-carbon unit generated from serine substantiates this view (Table 9). Glycine also acted as a source of C-1 units (Table 12) presumably by a complex reaction involving decarboxylation (Scheme 2; Scheme 4, reaction 2). The C-1 units generated in this reaction were oxidized or reduced to the formyl or methyl levels of oxidation respectively. When reduced FAD was supplied in the presence of homocysteine the synthesis of methionine was also observed.

Reactions involving glycine cleavage

The close relationships between CO_2, NH_3 liberation and serine synthesis from glycine by leaf mitochondria (Kisaki *et al.*, 1971), and the labelling of glycine from L-[3-^{14}C]serine by cotyledon mitochondria (Table 9), both indicate that a direct cleavage of glycine is catalyzed by plant mitochondria. Combined with the more definitive enzyme studies (Tables 13 to 16 and Figures 5 to 8) these lines of evidence are consistent with the scheme of glycine catabolism which has been proposed by other workers for rat liver (Yoshida and Kikuchi, 1970) and *Peptococcus glycinophilus* (Klein and Sagers, 1965a,b, 1966a,b).

The stimulation of the bicarbonate exchange reaction by pyridoxal-5'-phosphate and dithiolthreitol (Table 14) and the saturating substrate concentrations for glycine and bicarbonate, apparent K_m values of 1.8 mM and 12.5 mM respectively, (Figure 7), suggest strong similarities to the reaction mechanisms proposed for other tissues (Scheme 2). The effects of NAD concentrations on the cleavage reaction (Figure 7) further implies some common reaction mechanisms.

The first reaction of glycine cleavage (Scheme 2, reaction 1) which is characterized by the exchange of the carboxyl group of glycine with bicarbonate was found to require pyridoxal-5'-phosphate and mitochondrial protein in the presence of isotonic sucrose for optimum activity (Table 14). The involvement of more than one protein in this exchange reaction in higher plants still remains obscure but as disruption of mitochondrial integrity reduced enzyme activity (Table 15) it may be concluded that more than one protein was involved in this reaction.

The second reaction (Scheme 2, reaction 2) requires NAD^+, H_4PteGlu, pryidoxal-5'-phosphate and mitochondrial fraction for maximal activity in the absence of bicarbonate (Figure 7C). These requirements imply that additional protein units are utilized in a more complex reaction resulting in the complete cleavage of glycine. The synthesis of glycine from [3-^{14}C]serine (Table 9) further indicates that this latter reaction may be reversible in pea mitochondria. However, an alternate explanation for the latter observation might involve the decarboxylation of serine and subsequent conversion of ethanolamine to glycine. If glycine cleavage is freely reversible as observed for rat liver mitochondria (Sato *et al.*, 1969) then it is unlikely that the free energy of the reaction would be directly utilized in ATP

synthesis and stimulated by ADP in pea mitochondria as suggested by Bird *et al.* (1972) for leaf tissue. Such carboxylation reactions have not been found to require ATP for maximal synthetic activity in mammalian systems (Sato *et al.*, 1969).

The physiological significance of glycine cleavage in pea cotyledon mitochondria may be different from other tissues examined. During seed germination considerable proteolysis and ultrastructural degradation occurs in the cotyledon (Bain and Mercer, 1966). Hydrolysis of the storage protein releases substantial amounts of serine and glycine (Lawrence and Grant, 1963). Serine and consequently glycine can also be formed by the conversion of glycollate into these amino acids (Cossins and Sinha, 1967; Tanner and Beevers, 1965). The subsequent catabolism of glycine and serine by glycine decarboxylase may yield other products necessary for seed germination.

The affinity of this enzyme for glycine, apparent K_m value of 1.8 mM (Figure 7), would be prerequisite for the decarboxylation of glycine produced in the cotyledon by protein hydrolysis or other mechanisms. Decarboxylation could yield one CO_2, an active C-1 unit and one reduced pyridine nucleotide (Tables 12, 14 and Figure 7). After the initial reaction, the C-1 unit produced could be reduced or oxidized (Table 12) for utilization in the mitochondrial pteroyl-glutamate pool. Several alternate pathways for the utilization of this C-1 unit exist in the mitochondria. First, 5,10-CH_2-H_4PteGlu produced upon decarboxylation may be oxidized to the formyl level of oxidation yielding another reduced pyridine nucleotide (Tables 8 and 12). Utilization of 10-HCO-H_4PteGlu for formylation reactions such as the synthesis of formyl methionine may occur. The synthesis of the latter

compound must be integral to the development upon germination of the pre-existing respiratory capacities in this tissue (Solomos *et al.*, 1972). A second alternate pathway might involve complete oxidation of 10-HCO-H_4PteGlu to CO_2 (Scheme 4, reaction 8) (Table 17). This reaction could yield more energy in the form of another reduced pyridine nucleotide (Kutzbach and Stokstad, 1968).

The metabolic fate of the C-1 unit from serine and glycine entering the mitochondrial pteroylglutamate pool at the hydroxymethyl level of oxidation is likely determined by the physiological demands upon and within the mitochondria unless regulation occurs upon entry to the pteroylglutamate pool. In this regard the inhibition of glycine decarboxylase by L-methionine may be significant. When methionine concentrations in the mitochondria increase direct inhibition of the *de novo* generation of methyl groups may occur (Figures 9 and 11).

Serine hydroxymethyltransferase, though generally assumed in plants and lower organisms to be a primary source of C-1 units, has not been clearly shown to be regulated by 5-CH_3-H_4PteGlu or methionine and its metabolites. The nature of the structural or compartmental limitations on this enzyme in higher plants may however preclude such types of regulation.

Methionine biosynthesis

The present study has implicated the mitochondria in the biosynthesis of various amino acids related to one-carbon metabolism, such as the *de novo* synthesis of methionine. The data presented (Tables 18 and 19) illustrate that the 5-CH_3-H_4PteGlu homocysteine-dependent methyltransferase present in pea cotyledons may be largely

compartmented within the mitochondria. Several properties of this enzyme were found to be similar to the enzyme partially purified directly from cotyledon homogenates.

The synthesis of L-methionine by a mitochondrial methyltransferase utilizing 5-CH_3-H_4PteGlu$_3$ as the methyl donor has been observed for rat liver (Wang *et al.*, 1967) though the presence of S-adenosylmethionine: L-homocysteine S-methyltransferase could not be detected. In contrast, the latter enzyme was detected in the ammonium sulphate fraction of isolated mitochondria utilized in the present study (Tables 18 and 19) thus, indicating some role for this enzyme in mitochondrial one-carbon metabolism.

The presence of substantial levels of 5-CH_3-H_4PteGlu transmethylase in mitochondria (Table 18), and its high affinity for the methyl donor (apparent K_m for 5-CH_3-H_4PteGlu of 25 μM), implies that a very rapid turnover of methyl groups occurs within this organelle. Furthermore, the presence in the mitochondria of several enzymes known to be involved in the biosynthesis of homocysteine (Tables 21 and 22) and the apparent binding of methylated derivatives of H_4PteGlu to the mitochondria (Table 6) further supports this contention.

Product inhibition by L-methionine of the 5-CH_3-H_4PteGlu transmethylase (Figure 11) as well as other mitochondrial reactions involved in the generation of C-1 units through the pteroylglutamate pool would no doubt result in strict regulation of the *de novo* production of methionine during the germination of this seed, especially as this process may be augmented somewhat by the extensive hydrolysis of reserve protein during germination (Lawrence *et al.*, 1959).

The ability of isolated pea mitochondria to activate methionine in the synthesis of SAM (Table 20) is consistent with the observation by Davies (1966) that L-methionine inhibits mitochondrial respiration by combining with ATP to form SAM. SAM produced in the mitochondria may then be utilized in other methylation reactions, perhaps also similarly compartmented.

In the latter regard, it is known that the restriction and modification of DNA (recently reviewed by Meselson *et al.*, 1972) is highly specific, utilizing a specific methylase for the methylation of different bases as well as utilizing SAM as the methyl donor. The degree or activity of these specific transmethylation reactions in the development of the mitochondrial electron transport chain (Solomos *et al.*, 1972) could be facilitated by a mitochondrial synthesis of SAM (Table 20).

Concluding remarks

In conclusion, it has been shown that the mitochondria of this tissue possess enzymes necessary for extensive C-1 metabolism. In this respect, these organelles may be quite autonomous. Furthermore, the intracellular localization of pteroylglutamate derivatives implicates the mitochondria in physiological roles integral to the metabolism of the cell as a whole. It is not clear however whether these derivatives, synthesized within the mitochondria, are also utilized by the rest of the cell. It is possible that sufficient mitochondrial enzymic capacity can be detected to provide the amounts of pteroylglutamate derivatives, methionine and SAM, required to support syntheses of DNA, RNA and protein within the mitochondrion. These activities are in fact prerequisites for the development of mitochondria in higher organisms.

The nature of the processes which control production and utilization of individual pteroylglutamates within the mitochondrion are still largely unknown and warrant further detailed study.

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